HIGHLY MULTIPLEXED REPORTER CARRIER SYSTEMS

The present invention is generally in the field of detection of molecules, and specifically in the field of detection of multiple different molecules in a single assay.

It is an object of the present invention to provide a composition that permits the indirect detection of a large number of different analytes in a single sample or group of samples.

It is another object of the present invention to provide a composition that permits the indirect detection of a large number of different proteins in a single sample or group of samples.

BACKGROUND OF THE INVENTION

The analysis of proteins in histological sections and other cytological preparations is routinely performed using the techniques of histochemistry, immunohistochemistry, or immunofluorescence. By performing immunofluorescence with antibodies labeled with different colors, it has been possible to detect simultaneously 2, 3, or even 4 different antigens present in cellular material. In the future, time-resolved fluorescence may permit the extension of immunofluorescence methods to the detection of 6 to 12 different antibodies simultaneously. Likewise, RNA detection by fluorescence in situ hybridization permits the detection of 2 to 4 different RNAs in cellular material, and it may also be extended to permit the detection of 6 to 12 different RNAs by time-resolved fluorescence.

There is a need for a sensitive method that will permit the cytological detection of larger numbers of proteins or RNAs simultaneously. Theoretically, the simultaneous measurement of the concentration of 20 to 50 different protein (or RNA) species should be highly informative as to the specific status of dynamic cellular processes in normal development, in stages of disease, in response to drug treatment or gene therapy, or as a result of environmental exposure or other deliberate or inadvertent interventions.

The study of cells by measuring the identity and concentration of a relatively large number of proteins simultaneously (referred to as proteomics) is currently a very time-consuming task. Two-dimensional (2D) gel electrophoresis

is the most powerful tool for studying the expression of multiple proteins, but this technique is not readily adaptable to in-situ cell analysis. Typically, many thousands of cells are required to perform a single 2D gel analysis. In order to identify different protein expression profiles in heterogeneous tissue samples, one would need the capability to analyze the proteins expressed in a small number of cells. This capability is most relevant in the analysis of histological or cytological specimens that may harbor dysplastic or pre-malignant cells. Such cells, which may precede the development of cancer, need to be identified when present as small foci of 10 to 50 cells, before they have a chance to give rise to tumors. Unfortunately, the amount of protein obtained from 10 to 50 cells is insufficient for 2D gel analysis, and is problematic even with the use of radioisotopes to label the protein.

Mass spectroscopy is another powerful technique for protein analysis. However, the direct analysis of proteins present in samples containing small numbers of cells is not possible with prior mass spectroscopy technology, due to insufficient sensitivity. A minimum of 10,000 cells is required for mass spectroscopic analysis of tissue samples using prior technology.

Current methods for the analysis of microarray hybridization experiments rely on the use of a two-color signal readout system. For example, Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-70, describe an experiment where cDNA prepared from one tissue is labeled with the dye cy3, while cDNA from another tissue is labeled with the dye cy5. After the labeling reactions are performed, the two labeled DNAs are mixed, and hybridized by contacting with the surface of a glass slide containing a cDNA microarray on its surface. At the end of the hybridization reaction, the microarray surface is washed to remove unhybridized material, and the glass slide is scanned in a confocal scanning instrument designed to record separately the cy3 and the cy5 fluorescence intensity, which is saved as two different computer files. Computer software is then used to calculate the fluorescence ratio of cy3 to cy5 at each of the specific dot-addresses on the DNA microarray.

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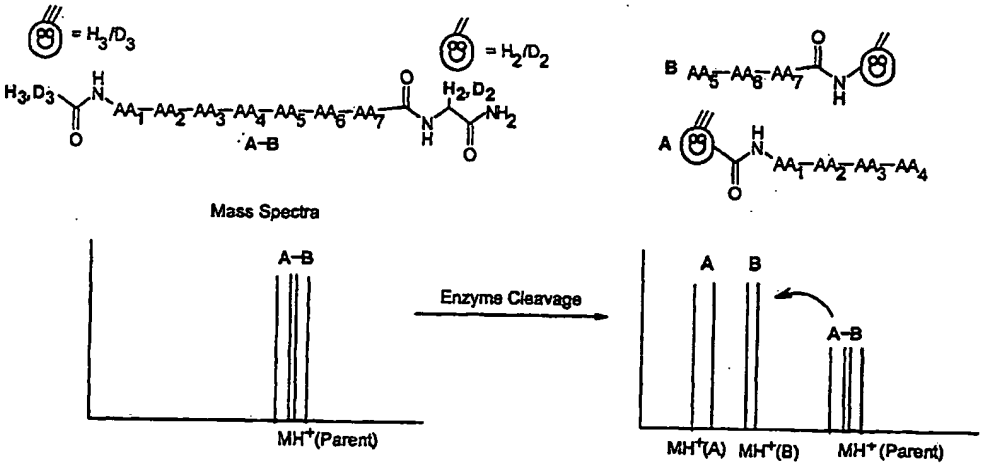
(54) Title: METHOD OF MASS SPECTROMETRY



(57) Abstract: A method of analysing the cleavage of a polymer wherein the polymer is differentially labelled with an isotope and the cleaved fragments of the polymer are analysed by mass spectrometry is disclosed. The method is particularly useful for analysing the cleavage of proteins and more particularly for determining substrate specificity for enzymes such as proteases.

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METHOD OF MASS SPECTROMETRY

Field of the invention

The present invention relates to methods of analysing cleavage of a polymer wherein the polymer is differentially labelled with an isotope and the cleaved fragments of the polymer are analysed by mass spectrometry. In particular it relates to methods of determining a cleavage site in the polymer, screening methods and methods of obtaining information about the structure and sequence of cleaved polymers.

Background of the invention

Chemical labels are widely used in chemical analysis. Among the types of molecules used are radioactive atoms, fluorescent reagents, luminescent reagents, metal-containing compounds, electron absorbing substances and light absorbing compounds. Such chemical labels may be covalently attached to the target to enable the substance to be detected. However, chemical moeities present on the target surface may interfere with the detection of the label. Such labels have been used in methods to identify the substrates of enzymes and their cleavage sites. However, such labels may have further limitations since the labels themselves can have an influence on the cleavage kinetics. There is therefore a need for a method of labelling potential substrates for such cleavage analysis which involves minimal perturbation of the structure of the substrate, presenting the substrate molecule almost indistinguishable from its unlabelled state.

In recent years, the approach of mass spectrometry has been used in the identification of proteins. Proteins are usually identified by the best match between the measured and calculated m/z (mass to charge ratio) values of the peptides. This approach however, encounters a number of problems such as low sample availablility, the presence of background or artifact ions and postsynthetic modification of proteins. Ultrahigh accuracy instruments may be required to determine absolute m/z values.

Recently, a more accurate approach has been reported wherein stable isotopic labels are incorporated into proteins. Uniformly ^{15}N-labelled proteins have been generated for improved accuracy of mass measurements by mass spectrometry (Jensen *et al* Anal Chem 71: 2076-2084 (1999)). Specific amino acids have been labelled with stable isotopes for incorporation into proteins so that these proteins may be distinguished from other molecules in a mass spectrum (Chen *et al* Anal Chem 72: 1134-1143 (2000)).

^{18}O labels have been used in the identification of C terminal peptides of proteins (Kosaka *et al* Anal Chem 72:1179-1185 (2000)).

Summary of the invention

The present invention provides a method of analysing cleavage of a polymer, the method comprising:

(a) providing a sample of said polymer, a portion of said polymer molecules having been labelled at a position on one side of a potential cleavage site with a first isotopic label and a portion of said polymer molecules having been labelled at a position on the opposite side of the potential cleavage site with a second isotopic label,

(b) incubating said sample under conditions suitable for cleavage at said potential cleavage site, and

(c) analysing the mass(es) of any cleaved fragments by mass spectrometry and thereby determining whether and/or where cleavage has taken place.

Preferably, the mass change caused by the presence of the first isotopic label compared with the mass in the presence of the normal element(s) at that position is different to the mass change caused by the presence of the second isotopic label compared with the mass in the presence of the normal element(s) at that position,

The present invention further provides a method of screening a library of polymers for cleavage, the method comprising:

(a) providing a library comprising two or more polymers labelled as described in claim 1,

(b) incubating said library of polymers under conditions suitable for cleavage, and

(c) analysing the mass(es) of any cleaved fragments by mass spectrometry and thereby determining whether and/or where cleavage has taken place.

The sample may be incubated with an enzyme under conditions suitable for the cleavage of a substrate.

In a further aspect of the invention, the method further comprises the step of quantifying the amount of the polymer or cleaved fragment(s) present after cleavage.

In another aspect of the invention, said polymer further comprises a detectable non-isotopic label on one side of the potential cleavage site and an inhibitor for said label on the opposite side of the potential cleavage site, and the method comprises the

additional step of detecting said detectable non-isotopic label, wherein the detection of said label indicates that said polymer has been cleaved.

The methods of the present invention may further comprise determining the structure or sequence of said polymer or of one or both of the cleaved fragments, optionally by further cleaving these fragments and determining the masses of these further fragments using mass spectrometry.

The present invention further comprises a kit for screening a library of polymers for cleavage, comprising two or more differentially isotopically labelled polymers as described above. The invention also comprises a kit for the preparation of such a polymer, comprising a set of chemical monomers which comprises monomers which have been isotopically labelled and monomers which have no isotopic label, in a form suitable for polymer synthesis.

Brief description of the drawings

Figure 1: Sample mass spectra for uncleaved (A-B) and cleaved (A, B) peptides labelled with either: Ⓓ = D2 or H2 Gly, or Ⓗ = D3 or H3 acetate.

Figure 2: Mass Spectra before and after cleavage of Ⓓ-Gly-Pro-Arg-Ala-Ala-Ala-Gly-Ⓓ with trypsin.

Figure 3: Mass spectrum of peptide 4.

Figure 4: Mass spectrum of peptide 4 following treatment with trypsin.

Figures 5a and b: Mass spectra of peptide 4 following treatment with NEP.

Figure 6: Summary of sample method used to screen peptides (A-B) for cleavage by an enzyme, isolation of peptide fragment A and any remaining peptide A-B and identification of their peptide sequences. MS1 and MS2 = isotopic labels.

Figure 7: Determination of optimal ratio of fluorescent group (F) to quencher (Q).

Figure 8: Mass spectrum analysis for resin 16.

Figure 9: Mass spectrum analysis for resin 17.

Figure 10: Mass spectrum analysis of resin 15 following treatment with trypsin

Figure 11: Strategy for synthesis of a library of differentially isotopically labelled compounds.

Figure 12: Mass spectra of compound $7_{(2)}$ (bj00080/2), compound $7_{(17)}$ (bj00083/5), compound $7_{(24)}$ (bj00014/9), and compound $7_{(31)}$ (bj00014/13) respectively.

Figure 13: A Mass spectrum following incubation of compound $7_{(2)}$ with trypsin: cleaved sequence H2N-Ala-Asn-Ile-Asp-Phe-Ala-Lys(ac)-NH2 (819/822).
B, C: Mass spectra of compound $7_{(17)}$ with trypsin: **B:** cleaved sequence Ac-Gly-Ala-Ala-Phe-Lys-Arg-OH (691/693), **C:** cleaved sequence Ac-Gly-Ala-Ala-Phe-Lys-OH.

Figure 14: Mass spectra of compounds $8_{(1-4)}$.

Figure 15: Mass spectra of compounds $8_{(5-8)}$.

Figure 16: Mass spectra of compounds $8_{(1-4)}$ following treatment with trypsin.

Figure 17: Mass spectra of compounds $8_{(5-8)}$ following treatment with trypsin.

Figure 18: Amino acid sequencing via mass spectrometry on peptide fragment differentially labelled with a stable isotope (⊛) at one terminal. Mass spectra show sample pattern produced by cleavage of amino acids from either the C or N-terminal of the peptide.

Detailed Description of the Invention

A series of methods for analysing the cleavage of a polymer have been developed based on the differential isotopic labeling of the polymer. These methods may be useful in discovering new or improved synthetic substrates for for both known and unknown enzymes, for example novel enzymes identified from the human genome. The methods may be used to identify an event of cleavage, the site of cleavage and additionally the sequence origin.

The methods of the present invention may also, for example, be used in screening methods to identify new substrates for enzymes, in positional peptide scanning libraries, in *in vitro/ex-vivo/in vitro* peptide tracking, in assaying methods, for oligonucleotide or peptide sequencing or in the measurement of differential protein expression.

Differentially isotopically labelled polymers

The present invention is based on the use of an isotopic label in the analysis of cleavage of a polymer.

The term "polymer" refers to any molecule made up of discrete "components". A suitable polymer for use in a method of the present invention may be any polymer which can be cleaved. A suitable polymer may, for example, be a biological polymer such as a peptide, polypeptide, protein, polynucleic acid (e.g. DNA, RNA or LNA/DNA/RNA

hybrid), lipid or carbohydrate. In a preferred aspect of the invention, the polymer comprises a peptide or protein.

The term "component(s)" refers to monomers which make up the polymer, e.g. amino acids (for proteins), nucleic acids (for DNA or RNA), sugars (for complex carbohydrates) and fatty acids (for lipids). A polymer will comprise 3 or more such components. A polymer may comprise, for example, 3, 5, 10, 20, 50, 100, 500 or more such components. A suitable polymer may be an oligomer, such as an oligonucleotide or oligopeptide. Preferably, the polymer is a linear polymer.

For use in the methods of the present invention, a sample of a polymer is differentially isotopically labelled. A sample of the polymer is partially labelled on either side of a potential cleavage site with a first and a second isotopic label such that a portion of said polymer molecules are labelled at a position on one side of the potential cleavage site with a first isotopic label and a portion of said polymer molecules are labelled at a position on the opposite side of the potential cleavage site with a second isotopic label. The two labelled portions are not mutually exclusive, i.e. some polymer molecules may fall within both portions and may therefore comprise both a first and a second isotopic label.

Such a sample may therefore comprise a mixture of polymer molecules which are unlabelled, polymer molecules which have only the first isotopic label, polymer molecules which have only the second isotopic label and polymer molecules which have both isotopic labels. Other suitable samples may comprise a mixture of polymer molecules which are unlabelled and polymer molecules which have both isotopic labels or a mixture of polymer molecules which have only the first isotopic label and polymer molecules which have only the second isotopic label. A suitable sample may comprise any combination of unlabelled, singly-labelled or doubly-labelled polymer molecules as long as the sample comprises both the first and second isotopic label.

By partially labelling in this way at each of these sites, each cleaved fragment will show a characteristic mass spectrum of two peaks representing the different isotopes present. The precise pattern of the mass spectrum will depend upon the relative amounts of the individual isotopes in the sample. Two fragments formed by cleavage of a polymer will therefore show a distinctive pattern of two pairs of mass peaks as described below.

A suitable sample may be 50% differentially labelled with isotopic labels on both sides of the potential cleavage site. That is, 50% of the polymer molecules in a sample

are labelled at a position on one side of the potential cleavage site with the first isotopic label, and 50% of the polymer molecules in the sample are labelled at a position on the opposite side of the potential cleavage site with the second isotopic label. Such a sample may therefore comprise only polymer molecules which have either the first or the second isotopic label, or may comprise any combination of unlabelled, singly-labelled and doubly-labelled polymer molecules as described above. If a given atom is labelled such that 50% of the atoms are of one isotopic form and 50% are of another isotopic form, the mass spectrum of the mixture will show a characteristic doublet in which the peaks are of approximately equal height.

A suitable sample comprises polymers having isotopic labels on either side of a potential cleavage site. For use in a method of the present invention, a polymer will be labelled at specific positions on each side of the cleavage site. Preferably, an isotopic label will be located on a single monomer within the polymer. A polymer may be labelled, for example, by substitution of a monomer by a labelled monomer, by addition of a labelled monomer or by addition of labelled moeities within or at an end of the polymer molecule. For example, if the polymer is a peptide, it may be labelled by substituting an amino acid by a labelled amino acid, by addition of a labelled amino acid to the polymer or by addition of a labelled moeity to the peptide, such as by acetylation, hydroxylation, carboxylation or phosphorylation.

Suitable positions for labelling may be determined for individual polymers based on, for example, the location of potential cleavage sites or the particular isotopic labels used. If the cleavage site is known, the isotopic labels can be positioned in the polymer on the appropriate sides of that cleavage site. For example, each isotopic label may be present at a terminal end of the molecule, adjacent to the cleavage site itself or at any intermediate location. If the potential cleavage site is unknown, the isotopic labels may, for example, be positioned towards the terminal ends of the polymer. If the polymer is a peptide, the isotopic labels may therefore be present in the region of the terminal amino acids, such as in the terminal five amino acids, or in moeties attached to these. The isotopic labels may preferably be present in the terminal monomers themselves, e.g. if the polymer is a peptide, the isotopic labels may be present in the terminal amino acids. Alternatively, the isotopic labels may be present in moeties attached to the polymer, preferably to the terminal monomer of the polymer. For example, the isotopic label may be introduced by acetylation at the amino terminus of a peptide.

A suitable isotopic label may comprise one or more chemical isotopes of one or more atoms present within the unlabelled polymer. An isotope of an element will have an identical number of protons and electrons, but a different number of neutrons, and therefore a different molecular weight. For example, an isotope may have one additional neutron and therefore have an increased molecular weight by one mass unit. Preferably each isotope is substituted for the normal atom within the polymer. For example, ^{1}H may be substituted by ^{2}H (deuterium), ^{12}C by ^{13}C, ^{16}O by ^{18}O, ^{14}N by ^{15}N and/or ^{79}Br by ^{81}Br. One or more different isotopes may be substituted into a polymer. The same isotope may be substituted one or more times into a polymer. A suitable isotope will have a half-life which is sufficiently long to minimise the likelihood of isotopic decay during the method. Preferably the half-life is greater than the amount of time between incorporation of the isotope into the monomer and the analysis by mass spectrometry of any cleaved fragments. Preferably the isotope has a half life of at least one day, more preferably at least one week. Most preferably the isotope is a stable isotope such as an isotope which has a half life of many years or many hundreds or thousands of years.

The first and second isotopic labels may be the same or different and may comprise the same chemical isotopes or different chemical isotopes.

The first and second isotopic labels may lead to the same mass change in the labelled polymer. For example, two hydrogen atoms may be substituted by two deuterium atoms on one side of a potential cleavage site leading to a mass change in the polymer of two atomic mass units. On the other side of the potential cleavage site two more hydrogen atoms may be substituted by two deuterium atoms, also leading to an increase in mass of two atomic mass units. As explained below, as each position will only be partially labelled in a sample of polymer, this will lead to a distinctive mass spectrum for both the polymer and its cleaved fragments.

In a preferred aspect of the present invention, the mass change caused in the polymer by the presence of the first isotopic label compared with the mass in the presence of the normal element(s) at that position is different to the mass change caused in the polymer by the presence of the second isotopic label compared with the mass in the presence of the normal element(s) at that position. For example, two hydrogen atoms may be substituted by two deuterium atoms on one side of the cleavage site and three hydrogen atoms substituted by three deuterium atoms on the other side of the cleavage site. Alternatively, one ^{12}C atom may be substituted by a ^{13}C atom on one side of the

cleavage site and two hydrogen atoms substituted by two deuterium atoms on the opposite side of the cleavage site.

Where two or more cleavage sites exist in the polymer, additional isotopic labels may be incorporated. Sufficient isotopic labels may be incorporated such that one is present in each cleaved fragment. Each isotopic label should cause a different mass change in the polymer when compared with the mass in the presence of the normal element(s) at that position.

The synthesis of isotopically labelled polymers and the introduction of isotopic labels to polymers may proceed by way of common organic and biochemical reactions and techniques. A polymer may be labelled in a number of different ways, at one or more different molecular positions or with different isotopes, either singly or in combination. Such methods are well understood by persons skilled in the art, and include techniques such as those disclosed in Ott, D.G., Syntheses with Stable Isotopes of Carbon Nitrogen and Oxygen, John Wiley & Sons, 1981, the disclosure of which is included herein in its entirety by way of reference. An isotopic label may be incorporated into a monomer which is then introduced into the polymer molecule. For example a particular labelled amino acid may be incorporated into a protein during cell growth or in an *in vitro* transcription/translation system as described in Yabuki *et al* (J Biomol NMR 11:295-306 (1998)).

In one embodiment the polymer(s) may be attached to a solid support. The solid support may take the form of, for example, beads, a solid surface, a solid substrate, particles, pellets, discs, capillaries, hollow fibres, needles, solid fibres, organic or inorganic gels or insoluble inorganic particles such as particles formed from fullerenes. Beads may be polymeric beads such as cellulose beads or resin beads. Resin beads may be produced from functionalised polymer resins such as polystyrene resins, polyacrylamide resins and dimethylacrylamide resins.

The polymer may be attached to a solid support by a linker molecule. Preferably the linker molecule is a cleavable linker molecule. Preferably the linker molecule is selectively cleavable, for example chemically cleavable, but is not cleaved by the method (e.g. enzyme) used in the screening assay.

Methods of analysing cleavage of a polymer

The methods of the present invention comprise the incubation of polymers under conditions suitable for cleavage.

The method of cleavage may be any method which leads to the cleavage of a polymer e.g. mechanical, chemical or enzymatic cleavage. In a preferred aspect of the invention, the polymer is incubated with an enzyme. For example, if the polymer is a polypeptide or protein, the enzyme may be a protease, if the polymer is a polynucleotide, the enzyme may be an endonuclease or exonuclease, if the polymer is a carbohydrate, the enzyme may be an amylase or glucanase and if the polymer is a lipid the enzyme may be a lipase. The polymer is incubated with an enzyme under conditions suitable for the cleavage of a substrate. Suitable incubation conditions will vary with the cleavage method, such as with the enzyme used, and may be easily determined by the person skilled in the art.

The mass of the cleaved fragments may be determined by any method known in the art. The mass should be measured sufficiently accurately that a molecular mass of each fragment may be determined. Preferably the mass should be measured sufficiently accurately that the mass change caused by the isotopic labels may be detected.

In a preferred aspect, the isotopic label(s) are detected by a method of mass spectrometry. Suitable methods of mass spectrometry include matrix-assisted laser-desorption ionization mass spectrometry, direct laser-desorption ionization mass spectrometry, electrospray ionization mass spectrometry, secondary neutral mass spectrometry and secondary ion mass spectrometry.

The cleaved fragments will show a distinctive pattern of two pairs of mass peaks (see Figure 1). Each fragment will be present in labelled and unlabelled form, each being seen as a separate peak. A fragment comprising an isotopic label will show a peak which is shifted with respect to the equivalent unlabelled fragment because of the different molecular weight of the isotope(s) compared with the normal element(s). This distinctive pattern allows easy determination of whether cleavage has occurred. The presence of one or two such pairs of peaks indicates that the polymer has been cleaved. The mass of each fragment may be determined from such a spectrum. This distinctive pattern of pairs of peaks representing labelled cleaved fragments facilitates the identification of the molecules of interest without the need to extract and purify them from the sample or reaction mixture.

In a preferred aspect the sample of polymer cleaved comprises unlabelled polymer molecules, polymer molecules comprising the first isotopic label only, polymer molecules comprising the second isotopic label only and polymer molecules comprising both the first and second isotopic labels.

If the first and second isotopic labels are the same, or are different but lead to the same mass change in the polymer, this will lead to a characteristic mass spectrum of three peaks. One peak corresponds to the unlabelled polymer, one to polymer containing only one isotopic label and the third peak corresponds to polymer containing both the first and second labels. As both labels lead to the same mass change in the polymer, polymer containing either the first or the second label will have the same molecular weight and these two types of molecule will be seen as a single peak on a mass spectrum.

If the first and second isotopic labels lead to a different mass change in the polymer, analysis of an uncleaved sample will show a characteristic mass spectrum of four peaks (see Figure 1). One peak corresponds to the unlabelled polymer, one to polymer comprising the first isotopic label but not the second (shifted with respect to the unlabelled polymer because of the different molecular weight of the isotope(s) compared with the normal element(s)), a third peak will correspond to polymer comprising the second isotopic label but not the first, and a final peak will correspond to polymer comprising both isotopic labels (shifted by the different molecular weights of both isotopic labels). As the first and second isotopic labels lead to a different mass change in the polymer, polymer molecules having only the first label will have a different molecular weight to polymer molecules having only the second label. Two separate peaks corresponding to polymer containing each of these labels should therefore be seen on a mass spectrum. The precise pattern will depend upon the relative amounts of the different isotopes.

In either of these cases, the distinction between the three or four peaks of the uncleaved polymer and the two pairs of peaks of the cleaved fragments make it possible to readily determine from a mass spectrum whether cleavage has taken place.

The methods of the present invention may be used to determine the specific location of a cleavage site within a polymer. In the embodiment wherein the first and second isotopic labels cause different mass changes when compared with the mass of the polymer in the presence of the normal isotope(s) at their positions, it can be determined from the spectrum which pair of peaks corresponds to which cleaved fragment. Each pair

of peaks will show a split equivalent to the difference in mass between a labelled and unlabelled fragment (see Figure 1). The individual cleaved fragments may therefore be identified from such a mass spectrum, their masses determined, the sequence established and the exact location of the cleavage site within the polymer may therefore be inferred.

If the polymer has been cleaved once, at a position between the two isotopic labels, the sum of the molecular weights of the cleaved fragments should be equal to the molecular weight of the uncleaved polymer.

If the sum of the molecular weights of the cleaved fragments is less than the molecular weight of the uncleaved polymer, this indicates that cleavage has also occurred elsewhere within the polymer. The location of other such cleavage sites may be determined by incorporating additional isotopic labels in the polymer as described above and analysing the cleaved fragments as described above; by determining the structure or sequence of the isotopically labelled fragments as described below; or by a combination of these two methods.

Data obtained by mass spectrometry may be analysed by any suitable software. For example, peak split recognition software ('cluster analysis') is available from Micromass© Ltd and has to date been used for analytical construct and bead decoding technologies (McKeown S.C., Watson, S., Carr R., Marshall, Tetrahedron Lett (1999) 40: 2407; Lane S.J., Pipe A.J., Rapid Communications in Mass Spectrometry (2000) 14: 782-793). Such software simplifies the data interpretation by filtering out specific 'peak splits'. Such software could also be used to automate the interpretation of the mass spectra obtained using the methods of the present invention.

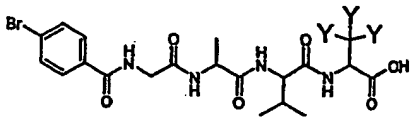
Quantification

Methods of the invention may also be used as part of an assaying technique based of mass spectrometry, for example by quantification of the amount of cleavage or the amount of the polymer or cleaved fragment(s) in a sample. For example, the amount of cleavage which has occurred following any of the methods of the invention described herein may be quantified.

The polymer may further contain a moiety which aids quantification. Suitable moieties would be well known in the art. Methods of quantification (and suitable moieties for use in such methods) include, for example, UV, Fluorescent and Visible

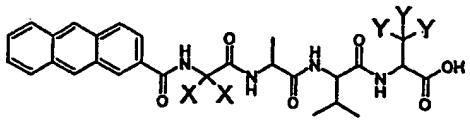
Spectroscopy (e.g. using *p*-nitrophenol,dansyl, aminomethylcoumarin), Inductively coupled Plasma Mass Spectrometry (ICP-MS), (e.g. using Chlorine, Bromine, Sulphur, Phosphorus), NMR, Weighing, Acoustic Photo Spectroscopy, Mass Spectrometry, Raman Spectroscopy, Atomic Absorption, Elemental Analysis, Electrolysis, Circular Dichroism, Elisa, ESR (using Electron Spin Resonance).

ICP-MS Handle

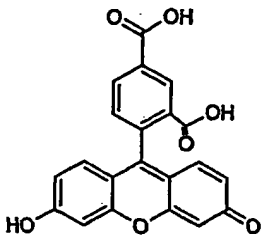


$^{79,81}Br$ supplies both isotope and quantitation label

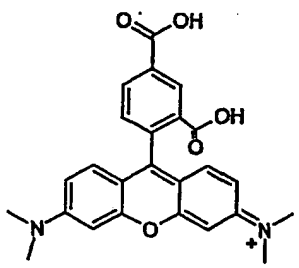
UV Fluorescence Handle



The polymer may also contain combined fluorescent-quench pairs (e.g. FRET pairs such as FAM/TAMRA):



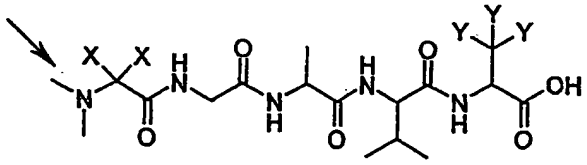
FAM



TAMRA

Additionally the polymer may contain a sensitising group to enhance detection by the Mass Spectrometer. For example to enhance a molecule to ESI-MS (positive mode) an amino group can be incorporated. For example:

MS sensitising Group



The polymer may additionally or alternatively comprise a moeity which may be used in the separation of the polymer or its cleaved fragments from other material. A suitable moeity may be one of a specific binding pair such as biotin. A suitable moeity may be removable from the polymer molecule, for example by specific cleavage. A

moeity may be present on one side of the potential cleavage site or on both sides of the potential cleavage site. The presence of such a moeity would allow for the polymer or its fragments to be more easily separated from the cleavage mixture, for example, using the other of the specific binding pair to capture polymer or cleaved fragments that comprise the moeity. The other of the specific binding pair may be bound to a solid support and may capture molecules comprising the moeity when these are incubated with or passed over the solid support. These binding pair complexes including the polymer or cleaved fragment may then be removed from the solid support.

More than one such moeity may be attached to a polymer molecule. The same moeity may be attached to the polymer molecule at more than one location. If the moeity is present on both sides of the potential cleavage site, it may be used to separate uncleaved polymer and any cleaved fragments from the cleavage mixture, for example from any enzymes or chemicals which are used to induce cleavage. Alternatively, two or more different moeities may be attached to the polymer molecule. For example, a polymer for use in a method of the present invention may comprise a different moeity on each side of the potential cleavage site. Each cleaved fragment may then be captured separately, for example by simultaneously or separately using the others of the specific binding pairs to capture each cleaved fragment. In this way the cleaved fragments may be separated from each other.

Screening methods

The methods of the invention may be used to screen a library of polymers for those polymers which are cleaved by a particular method, for example by a particular enzyme. The methods of the invention may, for example, be used to identify substrates for a particular enzyme, e.g. a protease. Potential substrates may be prepared by introducing isotopic labels as described above. The substrates may be prepared as single compounds or large libraries, e.g. 10^{10}. These individual or mixed substrates may then be incubated with one or more enzymes. The resulting fragments may then be analysed by mass spectrometry and the cleaved substrates identified.

Alternatively, the methods may be used to screen a library of enzymes for the ability to cleave a particular substrate.

The methods of the present invention may be used to monitor the cleavage of polypeptides or polynucleotides, for example, polypeptides or polynucleotides which

vary by one or more amino acids or nucleotides respectively at a site which effects the ability of the enzyme to cleave the substrate. The site may or may not be part of the recognition sequence for the enzyme. For example, for a polynucleotide, the cleavage site may be selected to be related to a specific sequence or nucleotide polymorphism, for example a single nucleotide polymorphism (SNP). The polypeptides or polynucleotides are differentially isotopically labelled as described above. They may then be treated to induce cleavage, preferably specific cleavage at the site of the variation e.g. by introducing an enzyme such as a protease (for polypeptides) or an endonuclease or exonuclease (for polynucleotides). Preferably the ability of the enzyme to cleave the substrate will depend on the sequence or nucleotide which varies between the substrates. The fragments produced may be analysed by mass spectrometry. The methods of the present invention may therefore be used to detect specific variants in the polypeptide(s) or polynucleotide(s). Such methods may further be linked to a molecular diagnostic assay technology. Such an assay may involve, for example, target amplification (e.g. PCR) or signal amplification (e.g. a branched DNA assay).

For any of the above screening approaches, the cleavage sites in such polymers may be determined by the methods described above, and the sequences of such molecules may be determined by methods as described below.

A suitable polymer for use in a screening method of the invention will comprise any differentially isotopically labelled polymer as described above. A suitable polymer may further comprise a detectable label on one side of the cleavage site and an inhibitor for that label on the opposite side of the cleavage site.

A suitable polymer may be synthesised by methods known in the art. For example, it may be produced by attaching a detectable label and an inhibitor to any of the polymers described above.

A suitable detectable label may be any label which may be reliably detected in a screening method. A detectable label may be a moiety which aids quantification or separation as described above. Preferably the detectable label is a chemical label. For example, suitable chemical labels may include radioactive atoms, fluorescent reagents, chromophores, luminescent reagents, metal-containing compounds, electron absorbing substances and light absorbing compounds. A suitable inhibitor will be one which prevents the detection of the detectable label. Preferably the inhibitor will inhibit the detection of the detectable label while in close proximity to the label (i.e. when the

polymer is intact) but will no longer inhibit the detection of the label when the polymer is cleaved. In a preferred aspect, the detectable marker is a fluorophore and the inhibitor is a quencher for that fluorophore.

Preferably, the ratio of detectable marker to inhibitor is optimised such that in the absence of cleavage the marker is not detectable, but on cleavage the marker becomes detectable (see Figure 7). An appropriate ratio of detectable marker to inhibitor will depend on the particular marker and inhibitor used and the method of detection. An appropriate ratio for a particular combination of label and inhibitor may be determined by the skilled person using methods known in the art.

A suitable polymer may be used in a screening method comprising the steps of incubating under conditions suitable for cleavage and screening for the detectability of the label. If the polymer is not cleaved, the inhibitor should prevent the detectability of the label. If the polymer is cleaved by the enzyme, the inhibitor will be separated from the label and the label should be detectable. The cleaved fragment comprising the label may then be identified and isolated. The cleaved fragment may be analysed using mass spectrometry as described above and its sequence or structure may be determined as described below.

In one embodiment the polymer is attached to a solid support. Any suitable solid support may be used, for example those solid supports described above. The polymer may be attached to the solid support via a linker molecule. Preferably the linker molecule is a cleavable linker molecule. Preferably the linker molecule is selectively cleavable, for example chemically cleavable, but is not cleaved by the method (e.g. enzyme) used in the screening assay.

Preferably the fragment which is cleaved from the support-linker-polymer construct during the assay comprises the inhibitor. The detectable marker may be attached to the polymer, between the polymer and any linker molecule, directly to any linker molecule, between a linker molecule and the solid support or it may be attached directly to the solid support. One or more polymers may be attached to a solid support.

In a preferred aspect the polymer is attached to a resin bead. For example, a library of polymers may be synthesised on resin beads that incorporate fluorescent and quenching groups. A number of molecules of each polymer may be synthesised on an individual bead. The library may be designed such that after polymer cleavage, the resulting bead emits a fluorescent signal on irradiation (see calculation of R_{foz} below).

This fluorescence signals the event of cleavage. The recognisable bead can be selected and further analysed. The cleaved (isotopically labelled) polymer, along with any remaining, uncleaved polymer, may be then released from a bead by specific cleavage of the linker. The released polymer(s) may then, for example, be subjected either to mass measurement, for example by mass spectrometry, to confirm the event of cleavage, to identify the site of hydrolysis as described above, or to further fragmentation to allow identification of their sequences as described below.

Substrate Optimisation

The methods of the present invention may be utilised in a number of methods to determine the optimal polymer substrate for a particular enzyme or other cleavage method.

The methods of the present invention may be used in positional scanning libraries, for example in positional scanning peptide libraries. A number of polymers (e.g. peptides) may be synthesised which vary by a single monomer (e.g. a single amino acid). These polymers are differentially isotopically labelled as described above. They may then be treated to induce cleavage, e.g. by introducing an enzyme, and the fragments produced analysed by mass spectrometry. The cleavage conditions may be varied and the data obtained analysed to determine the optimal monomers required in the polymer substrate for that cleavage method (e.g. enzyme).

A polymer of a given length may be systematically shortened to investigate the contribution of individual monomers to cleavage. Such a method may be used to find the shortest sequence of monomers which would still allow for a cleavage even to occur. Differentially isotopically labelled polymers as described above may be used in such a method. In such methods, the truncated permutations may be simultaneously synthesised producing a mixture of differentially isotopically labelled sequences. The ability to identify labelled fragments by their label and length as described above, rather than identifying the starting polymer which has been cleaved, makes it possible to perform cleavage on such a mixture of polymers together.

Tracking and Diagnostics

Differentially isotopically labelled polymers as described above may also be used in tracking experiments. For example, biological polymers such as peptides may be

differentially isotopically labelled as described above and the course of such polymers in a biological system tracked by analysis using mass spectrometry.

For example, such labelled peptides may be used for *in vitro / ex vivo / in vitro* peptide tracking. Biological or chemical degradation of such polymers may also be tracked by identifying the appearance of pairs of peaks by mass spectrometry indicating that the labelled molecule has been cleaved.

Such methods also find applications in diagnostics. A polymer which has been differentially isotopically labelled as described above may be introduced into a biological system, for example by administration to a patient, e.g. a human patient. The cleavage of the polymer may then be monitored by taking samples from the biological system. Where a particular enzyme is unusually expressed, or over-expressed, (e.g. in a disease state) the cleavage of its substrate may be identified by the appearance in a mass spectrum of a unique pattern of peaks. For such a method, the labelled polymer may be given as a single entity or as a mixture of labelled polymers for multi-diagnostic testing. For example, a number of substrates known to be cleaved by the enzyme of interest may be used or a number of substrates each known to be cleaved by a possible enzyme of interest may be used.

The differential isotopic labelling of the polymer in such *in vivo, in vitro* or *ex vivo* methods means that the polymer and any potential cleavage products do not need to be extracted and purified and that they can be easily distinguished from any similar or equivalent molecules which are naturally present in the system. Further, the use of isotopic labelling rather than traditional chemical labelling techniques reduces the possibility that the presence of the label will have an influence on the cleavage kinetics.

Polymer Sequencing:

If the structure or sequence of a polymer used in a method of the present invention is unknown (e.g. split-pool synthesis, deletion synthesis, pooled screening strategies) the polymer, or its cleaved fragments, may be subsequently (or simultaneously) sequenced by established methods. This may be used to identify a specific cleavage site and/or to obtain the sequence or structure of those polymers which have been cleaved.

As described above, individual cleaved fragments may be identified based on a mass spectrum. These fragments may then be further cleaved, for example chemically such as by Edman degradation, or by fragmentation using mass spectrometry techniques

such as MS/MS. Such methods of cleavage and fragmentation are known in the art. Analysis of the resultant fragments may be used to determine the structure or sequence of the pair of cleaved fragments. For example, if the polymer is a polypeptide, cleavage between individual amino acids will produce fragments of different sizes depending upon the particular amino acid removed. This data can be used to determine the primary structure of the original polypeptide.

Preferably, the cleaved fragments obtained by the methods of the invention are further analysed using mass spectrometry. Whereas a traditional mass spectrum obtained following fragmentation of a molecule reveals a fingerprint of single masses, the presence of differential isotopic labels offers additional ease of analysis of such fragments. Fragments still comprising an isotopic label may be identified by the presence of pairs of peaks on a mass spectrum. Fragments not comprising the isotopic label may be identified as single peaks on a mass spectrum. If the isotopic label was applied at a terminal of such a polypeptide, sequential cleavage of monomers, e.g. amino acids, from the opposite end of the molecule will result in a series of pairs of peaks on a mass spectrum. The difference in size of consecutive pairs will indicate the size of the monomer (e.g. amino acid) which has been removed, and this data may be used to determine the primary sequence of the original molecule. For example, in a peptide labelled at the N terminal, fragmentation in the C- to N- direction retains the peak split caused by the isotopic label giving rise to doublets, while the reverse fragmentation in the N- to C- direction produces only single mass peaks (see Figure 18). The use of mass spectrometry to determine the primary structure of a peptide is known in the art (e.g. McLuckey S.A. & Wells J.M., Chem Rev (2001) 101: 571-606; Biemann K., in Biological mass spectrometry, Proceedings of the Second International Symposium on Mass Spectrometry in the Health and Life Sciences, San Francisco, California, U.S.A., Elsevier Publishers: August 27-31 1989, p179).

Peak split recognition software ('cluster analysis') is available from Micromass© Ltd and has to date been used for analytical construct and bead decoding technologies (McKeown S.C., Watson, S., Carr R., Marshall, Tetrahedron Lett (1999) 40: 2407; Lane S.J., Pipe A.J., Rapid Communications in Mass Spectrometry (2000) 14: 782-793). This software simplifies the data interpretation by filtering out specific 'peak splits'. This

software could also be used to automate the interpretation of the mass spectra obtained using the methods of the present invention.

Kits

The invention further provides kits for use in the methods of the invention.

A kit may be provided for screening a library of polymers for cleavage, for example using a screening method of the invention. A kit may be provided for screening a library of polymers for those polymers which are cleaved by a particular method, for example by a particular enzyme. Such a kit may comprise two or more polymers which have been differentially isotopically labelled as described above. The polymers may be any polymers which are differentially isotopically labelled, for example polymers suitable for use in a method of the invention as are described above.

Suitable polymers for inclusion in a kit of the invention may be supplied in solid form, in liquid form, as a solution or on a solid support. A suitable solid support may be as described above. For example, a kit may comprise two or more resin beads, wherein attached to each bead is one or more molecules of a differentially isotopically labelled polymer as described above. Where polymers are attached to a solid support via a cleavable linker molecule, a kit may further comprise a linker cleaving reagent.

Suitable polymers may further comprise a detectable label on one side of the cleavage site and an inhibitor for that label on the opposite side of the cleavage site. Suitable polymers may comprise a moeity such as biotin which allows separation of the labelled polymer or cleaved fragment from other material.

The present invention also provides kits for the preparation of a polymer suitable for use in a method of the invention. Such a kit may comprise a set of chemical monomers which comprises monomers which have been isotopically labelled as described above. Such a kit will preferably contain monomers which comprise different isotopic labels so that different labels may be added either on each side of the cleavage site of the polymer, or to different polymers to be screened together. Such a kit may further comprise monomers that contain no isotopic label. Preferably the monomers are in a form suitable for polymer synthesis. A kit for the preparation of a polymer suitable for use in a method of the invention may comprise further components, for example means for combining monomers or for adding a monomer to a polymer molecule. For

example, a kit may further comprise suitable enzymes or buffers to enable polymerisation to occur.

In a preferred aspect of the invention, a kit for the preparation of a polymer suitable for use in a method of the invention comprises a set of isotopically labelled animo acids. Such a kit may further comprise a set of amino acids which contain no isotopic label. Such a kit may, for example, comprise all twenty proteogenic amino acids in unlabelled form and in one or more isotopically labelled forms. Such a kit may further comprise means for adding an isotopic label to an unlabelled amino acid, for example by modification such as acetylation, hydroxylation, carboxylation or phosphorylation. Such a kit may further comprise isotopically labelled and unlabelled chemical derivitising agents such as Ac2O-d6

In an alternative aspect, a kit for the preparation of a polymer suitable for use in a method of the invention comprises a set of isotopically labelled nucleotides. Such a kit may further comprise a set of nucleotides which contain no isotopic label. Such a kit may, for example, comprise a complete set of nucleotides in unlabelled form and in one or more isotopically labelled forms. Such a kit may further comprise means for adding an isotopic label to an unlabelled nucleotide, for example by modification such as acetylation, hydroxylation or carboxylation.

Examples

General Methods

Mass spectrometry analysis:

HPLC was run on a Varian 9010 instrument using a C_8 reverse phase column (Vydac, 22 cm, 0.5 cm ϕ) and 15 to 90% solvent B gradient (1 ml/min) as the mobile phase. [Solvent A: 1% TFA in water; solvent B; 0.5% TFA in MeCN:water (10:1), 20 min gradient time].

Mass Spectrometry measurements were carried out on a Micromass LCT System orthogonal accelerating Time-of-Flight (oa-TOF MS), in Positive Ion Electrospray mode (ESI,+ve)

Ionisation Mode: Positive Ion Electrospray (ESI,+ve)

Acquisition Mode: TOF MS

Scan Range:	100-1600 amu
Scan Rate:	1.0 sec, 0.10 Interscan delay
Flowrate:	0.400 ml min^{-1} (no flow split)
Runtime:	30 minutes.

Analysis of peptide purity:

The chromatography was carried out using a Waters Xterra column, (MSC_{18}, 2.1 x 150mm, 3,5µm) 0 to 100% solvent B gradient (0.4 ml/min) as the mobile phase. [Solvent A: 0.1% Formic Acid in water; solvent B; 0.1% Formic Acid in MeCN, 20 min gradient time]. A UV Diode Array (DAD) 190-600nm system was used for the detection.

Column :	Waters Xterra, MSC_{18}, 2.1 x 150mm, 3,5µm		
Channel A:	0.1% (%v) Formic acid (aq)		
Channel B:	100% (%v) Acetonitrile + 0.1% (%v) Formic acid (aq)		
Gradient:	0.00 mins	100 % A	0.00 %B
	2.00	100	0.00
	18.00	0.00	100
	20.00	0.00	100
	22.00	100	0.00
	30.00	100	0.00
Flowrate:	0.400 ml min^{-1}		
Detection:	UV Diode Array (DAD) 190-600nm		
Injection Volume:	5µl		

Trypsin was obtained from Sigma (reference T1426) and NEP was obtained from BioMep.

Example 1: Identification of cleavage site

Peptide A-B is an uncleaved molecule which is differentially labelled at both N- and C- termini with stable isotopes. ⊛ = two deuterium (D) or (H) atoms incorporated into Gly, ⊛ = three D or H atoms incorporated into acetate.

This uncleaved molecule shows a distinctive mass spectrum of four peaks due to the potential combinations of isotopes within the molecule (Figure 1).

Following cleavage to fragments A and B, two new pairs of peaks are seen on the mass spectrum (Figure 1). A pair of peaks with a peak split of 2 AMU (atomic mass units) indicates that the peptide has been cleaved and represents the C-terminal fragment B ( ). A pair of peaks with a peak split of 3 AMU indicates that the peptide has been cleaved and represents the N-terminal fragment A ( ). Any remaining uncleaved peptide A-B is seen as a remaining quartet of peaks at a higher relative mass. The site of cleavage is readily determined by the mass of the cleaved fragments A and B.

Example 2: Enzymatic cleavage of a peptide

The peptide Ac-Gly-Pro-Arg-Ala-Ala-Ala-Gly-NH_2 was synthesised. This peptide contains a trypsin cleavage site. This peptide was 50% differentially labelled with three deuterium atoms in place of three hydrogen atoms at the N terminus and with two deuterium atoms in place of two hydrogen atoms at the C terminus.

The substrate was incubated with trypsin and mass spectrum analysis performed directly on the 'incubation' mixture. Figure 2 shows the appearance of the two differential peak splits indicating the event of cleavage and secondly, from their individual 'peak split' state and masses, identifying its origin (C or N end) and cleavage site.

It has generally been found that since the peptides and deletion peptides synthesised in this manner all have a characteristic 'quartet', this may, in some instances, obviate the need to purify some synthesised peptides.

Example 3: Synthesis of 6 DiMaS peptides as isolated solids

Six differentially isotopically labelled (DiMaS) peptides were synthesised as described below. The peptides were selected to contain specific enzymatic cleavage sites.

i) Trypsin : **4** : AcGly-Pro-Arg-Ala-Ala-Ala-Gly-NH2

ii) α-Secretase: **6**: AcSer-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Gly-NH2

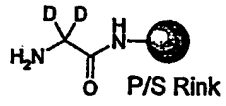
iii) β-Secretase : **8** : AcGly-Gly-Val-Val-Ile-Ala-Thr-Val-Ile-Gly-NH2

iv) γ secretase : **10**: AcHis-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Gly-NH2

v) Caspase 3: **12** : AcGly-Asp-Glu-Val-Asp-Gly-NH2

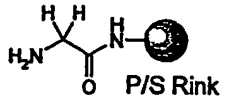
vi) Caspase 1 : **14**: AcTyr-Val-Ala-Asp-Ala-Pro-Val-Gly-NH2

Commercially available Rink amide resin (3g, 1.86 10^{-3} mol) was treated with 20% Piperidine in DMF and shaken for 1 h, the resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo. PyBop (4.84 g, 9.3 10^{-3} mol), DIPEA (3.2 ml, 1.86 10^{-2} mol), and Fmoc-gly-OH (D_2) (2.76 g, 9.3 10^{-3} mol) were mixed in NMP (15 ml) and added to the dried resin and shaken for 1h and then drained and washed as above. The resin was then treated with 20% Piperidine in DMF and shaken for 1 h, the resin was then drained and washed as above to give resin **1**.



1

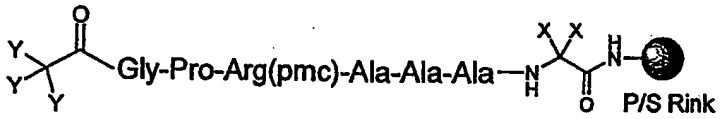
Commercially available Rink amide resin (3g, 1.86 10^{-3} mol) was treated with 20% piperidine in DMF and shaken for 1 h, the resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo. PyBop (4.84 g, 9.3 10^{-3} mol), DIPEA (3.2 ml, 1.86 E-2 mol), and Fmoc-gly-OH (2.76 g, 9.3 10^{-3} mol) were mixed in NMP (15 ml) and added to the dried resin and shaken for 1h and then drained and washed as above. The resin was then treated with 20% piperidine in DMF and shaken for 1 h, the resin was then drained and washed as above to give resin **2**.



2

Resins **1** (130 mg) and **2** (130 mg) were mixed and the sequence Gly-Pro-Arg-Ala-Ala-Ala was synthesised using an ABI433 No.1 Synthesiser using HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6-Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed

with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give **3**.



Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

3

Resin **3** (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 min then shaking at rt. for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1ml). The residue was then dissolved in H_2O (1ml) and freeze-dried to give a **4** as a white solid.

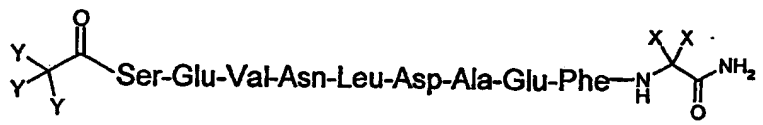
Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

4

HPLC/MS analysis of **4** is shown in Figure 3 (aj000260-8)

Resins **1** (130 mg) and **2** (130 mg) were mixed and the sequence Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe was synthesised using an ABI433 No.1 synthesiser using HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6-Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give **5**. Resin **5** (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 ins then shaking at rt. for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the

volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1ml). The residue was then dissolved in H_2O/dioxane and freeze-dried to give a **6** as a white solid.



Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

6

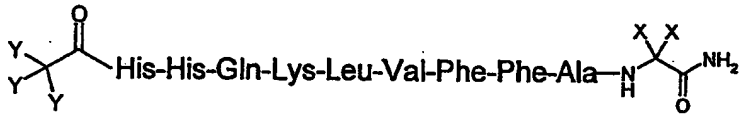
Resins **1** (130 mg) and **2** (130 mg) were mixed and the sequence Gly-Gly-Val-Val Ile-Ala-Thr-Val-Ile was synthesised using an ABI433 No.1 synthesiser using HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6-Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give **7**. Resin **7** (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 ins then shaking at rt. for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1ml). The residue was then dissolved in H_2O/dioxane (2 ml) and freeze-dried to give **8** as a white solid.

Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

8

Resins **1** (130 mg) and **2** (130 mg) were mixed and the sequence His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala was synthesised using an ABI433 No.1 synthesiser using

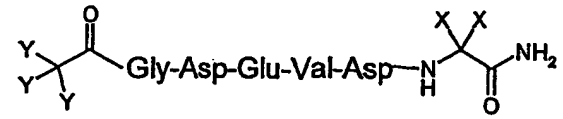
HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give **9**. Resin **9** (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 mins then shaking at room temperature for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1ml). The residue was then dissolved in H_2O/dioxane (2 ml) and freeze-dried to give a **10** as a white solid.



10

Resins **1** (130 mg) and **2** (130 mg) were mixed and the sequence Gly-Asp-Glu-Val-Asp was synthesised using an ABI433 No.1 Synthesiser using HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6-Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give resin **11**. Resin **11** (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 mins then shaking at room temperature for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1 ml). The residue was then dissolved in H_2O (1ml) and freeze-dried to give a **12** as a white solid.

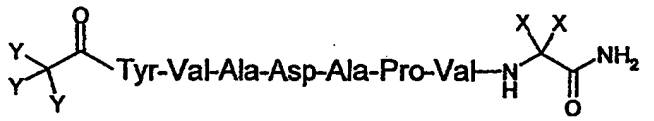
27



Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

12

Resins 1 (130 mg) and 2 (130 mg) were mixed and the sequence Tyr-Val-Ala-Asp-Ala-Pro-Val was synthesised using an ABI433 No.1 Synthesiser using HBTU/Fmoc protocol and standard side chain protection group strategy. A portion of the final amino resin (50 mg) was then treated with acetic anhydride (containing 50% D_6-Ac_2O) (0.2 ml) in 1:1 DCM/DMF (1 ml) and shaken for 2 h. The resin was then drained and washed with DMF (3 x 5ml), DCM (3 x 5ml), DMF (3 x 5ml), DMF (3 x 5ml), Et_2O (3 x 5ml), DCM (3 x 5ml) and finally Et_2O (3 x 5ml) and dried in vacuo to give resin 13. Resin 13 (50 mg) was cleaved by adding 3:0.2 TFA/TIPS (1ml) at 0 C for 10 mins then shaking at room temperature for 2 h. The resin was drained into a flask, washed with DCM (2 x 0.5ml) and the volatiles evaporated in vacuo. The residue was redissolved in DCM (1 ml) and again evaporated to remove the last traces of TFA (this procedure was repeated five times). The residue was then trituated with Et_2O (5 x 1 ml). The residue was then dissolved in H_2O and freeze-dried to give a 14 as a white solid.



Y= 1:1 H_3:D_3 X = 1:1 H_2:D_2

14

Example 4: Incubation of peptides with NEP and Trypsin

Incubation with Trypsin.

The peptide (10 µl of 1mM in Acetonitrile) was treated with a solution of buffer (50 mM HEPES, 150 mM NaCl and1 µM Zn^{2+}, 35 µl), and finally Trypsin was added (5µl, 2.5µM). The reaction was allowed to take place at room temperature for 9h and an aliquot of solution was removed for LC-MS analysis.

Incubation with NEP.

The peptide (10 µl of 1mM in Acetonitrile) was treated with a solution of buffer (50 mM HEPES, 150 mM NaCl and1 µM Zn^{2+}, 35 µl), and finally neutral endopeptidase (NEP) was added (5µl, 2.5µM). The reaction was allowed to take place at room temperature for 9h and an aliquot of solution was removed for LC-MS analysis.

Peptides **4** and **10** were incubated with trypsin as described above and then analysed by HPLC/MS to give masses according to Table 1.

Peptides **4**, **6** and **10** were incubated with NEP as described above and then analysed by HPLC/MS to give masses according to Table 1.

Table 1

Peptide	Incubation Enzyme	Relevant Masses (M+H or M+Na) (ignoring natural ^{13}C abundance)	Figures
4	No Incubation	640,642,643,645	3 (aj000260-8)
6	No Incubation	1121,1123,1124,1126	
8	No Incubation	926,928,929,931	
10	No Incubation	1225,1227,1228,1230	
12	No Incubation	632,634,635,637	
14	No Incubation	832,834,835,837	
4	Trypsin	371,374: AcGlyProArgOH 310,312: H_2NalaAlaAlaGlyNH$_2$ (Na+)	4 (aj000290-19)
10	Trypsin	591,594 AcHisHisGlnLysOH 652,654: H_2NleuValPhePheAlaGlyNH$_2$	
4	NEP	442,445 : AcGlyProArgAlaOH 239,241 : H_2NAlaAlaGlyNH$_2$ (Na+)	5a (aj000290-23) 5b (aj000290-23a)
6	NEP	650,652 : H_2NleuAspAlaGluPheGlyNH$_2$	

10	NEP	704,707 : AcHisHisGlnLysLeuOH 315,317 : NH_2PheAlaGlyNH_2	

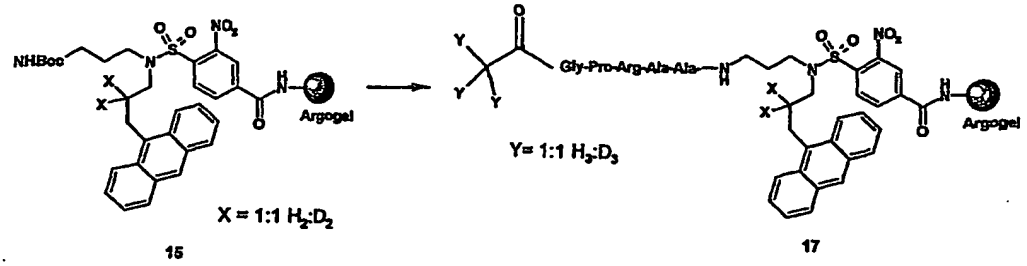
Example 5: Fluorescent On-Bead Peptide Screening

A library of peptides may be synthesised on resin beads that incorporate fluorescent and quenching groups. This library may then be used to screen for peptides which are cleaved by a particular enzyme (Figure 6). The library may be designed such that after peptide cleavage with an enzyme, the resulting bead emits a fluorescent signal on irradiation. This fluorescence signals the event of cleavage. The recognisable bead can be selected and further analysed. The ratio of fluorophore (F) and quenching agent (Q) can be optimised to give zero emission before incubation and a positive fluorescence on hydrolysis of the peptide (R_{foz} see Figure 7). This is important since too much initial quenching agent could still prevent emission even when significant cleavage has taken place.

The cleaved (MS isotopically labelled) peptide is then released via the linker along with any remaining, uncleaved peptide and subjected to MS fragmentation, confirming cleavage, identifying their sequences and identifying the site of hydrolysis (Figure 6).

This approach is amenable to screening all the peptides in one vessel. For example, 1 g of resin beads can be incubated together with the enzyme, subsequently washed of assay impurities, irradiated and the fluorescent beads removed by hand or picked via robotic automation. The 'active' substrates are then identified as described above. 1 g of resin beads may contain approximately ½ million beads, making this approach attractive for diverse screening methods.

Example 6: Synthesis of polymer supported DiMaS peptide



Resin 15 was prepared in a similar method to that described in the literature: (G. M. Williams et al. Angew. Chem., Int. Ed. Eng. 2000, 39 (18), 3293. Congreve et al. Org lett 2001, 3(4), 507)

Resin 15 (50 mg, 0.43mmol/g) was treated with 95% TFA (aq) for 2h. The TFA was drained and the resin washed with DCM, DMF, DCM, 10% DIPEA in DMF, DCM. Each of the amino acids were assembled as follows: A preformed activated ester of the amino acid was formed by mixing the Fmoc amino acid (0.43mmol) with HATU (0.43mmol) and DIPEA (0.86mmol) in DMF (2ml) for 10 min, this was then added to the resin and shaken for 4 h. The resin was then drained and washed with DMF, DCM, DMF and DCM (all x5). 20% Piperidine in DMF (5 ml) was then added to the resin and the resin shaken for 1h. The resin was then drained and washed with DMF,DCM,DMF,DCM (all 5 x 5 ml).

The sequence Gly-Pro-Arg-Ala-Ala was coupled on to the resin according to the above procedure. The resin was then treated with 10% Ac_2O/$Ac(D_3)2O$ (1:1) in DCM and shaken for 2h. The resin was then washed with DCM, DMF, DCM (all x5) to give resin **16**.

To remove all the pmc protecting group, the resin was shaken with 95% TFA (aq) for 2h. The resin was then drained and washed with DCM, 10% DIPEA in DMF, DCM to give resin 17.

A solution of Mercaptoethanol (0.1ml), DBU (0.33 ml) in Acetonitrile (1.5 ml) was prepared 30 mins before use. The resin (typically 50 beads) is treated with this solution (0.2 ml) and left to stand for 2h. The supernatant liquid is then removed and analysed via HPLC/MS.

The analysis for resin 16 is shown in Figure 8.
The analysis for resin 17 is shown in Figure 9.

Example 7: On-Bead Incubation of Supported DiMaS peptide

Incubation of resin with Trypsin.

The Resin 15 (ca. 20 beads) were treated with a solution of buffer (50 mM HEPES, 150 mM NaCl and1 C Zn^{2+}, 35 µl), and finally Trypsin was added (5µl, 2.5µM).

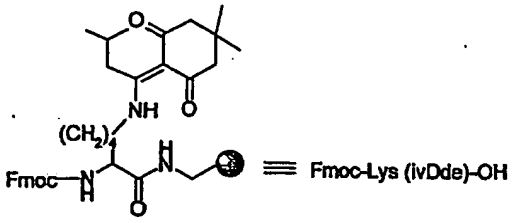
The reaction was allowed to take place at room temperature for 2h and an aliquot of solution was removed for LC-MS analysis. The analysis is shown in Figure 10.

Example 8 Preparation of DiMaS Tagged Peptide Libraries

A series of compounds were synthesised using the general strategy outlined in Figure 11.

Compound 1:

The Fmoc protected Rink Amide resin (3.900 g, 2.34 mmol) was treated with a 20% Piperidine solution in DMF (10 ml) for (2 x 10 min) and then washed with DMF (6 x 10 ml). The freshly prepared deprotected resin was then treated with Fmoc-Lys(ivDde)-OH (3.350 g, 5.85 mmol), HBTU (2.210 g, 5.85 mmol), and HOBt (0.790 g, 5.85 mmol) in DMF (10 ml). Finally, Hünig's base (1.510 g, 11.70 mmol) was added and the resin stirred at room temperature for 16 h. The resin was then washed successively with DMF (6 x 10 ml), dichloromethane (4 x 10 ml) and ether (2 x 10 ml), then dried *in vacuo* to give ***compound 1*** (4.600 g, 2.34 mmol) as a yellow solid. Kaiser test (Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.*, **1970**, 34, 595) was negative, Fmoc number 100%.

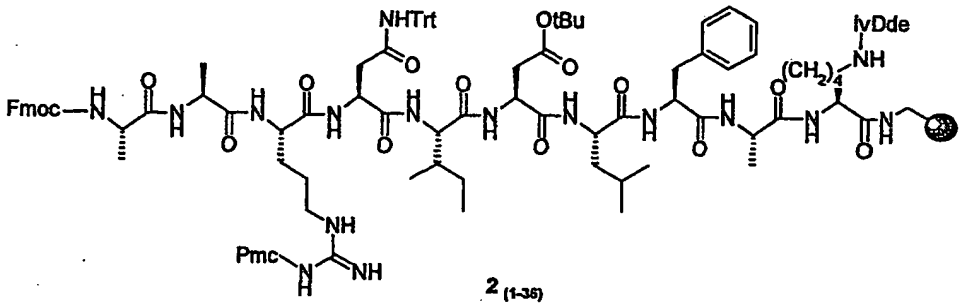


1

Compounds $2_{(1\text{-}36)}$:

The linear peptide was assembled on an automated ACT 357 MPS synthesiser using the standard protocol described by Sheppard[1]. The 36 resin aliquots were loaded manually into a standard (6 x 6) reaction block. The Fmoc protected Rink amide resin (0.12 g, 0.06 mmol) was treated with a 20% Piperidine solution in DMF (2 x 2 ml, 2 x 10 min) and then washed with NMP (10 x 2 ml). The deprotected resin was then treated with Fmoc-Ala-OH (0.056 g, 0.18 mmol) in NMP (700 µl) then HBTU (0.068 g, 0.18 mmol) and HOBt (0.024 g, 0.18 mmol) in NMP (180 µl). Finally Hünig's base (62 µl,

0.36 mmol) in DMF (360 µl) was added. The reaction block was agitated intermittently for 2 h after which the resin was drained under vacuum. A second coupling was performed using the same quantity of reagents as in the first coupling and finally the resin was washed with NMP (6 x 2 ml). The above procedure was repeated for the incorporation of Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ala-OH and Fmoc-Ala-OH respectively. The resin was finally washed with NMP (6 x 2 ml) then manually washed with DCM (2 x 2 ml) and ether (1 x 2 ml) then dried *in vacuo* to give **the protected peptide resin $2_{(1)}$** as a pale yellow solid (0.30 g, 100%). Kaiser Test was negative.



2 (1-36)

Compounds $2_{(2\text{-}36)}$ were prepared in an analogous manner to compound $2_{(1)}$ in the remainder of the wells in the ACT reaction block. The structures of compounds $2_{(1\text{-}36)}$ are given in Table 2.

Table 2: Structure of compounds $2_{(1\text{-}36)}$.

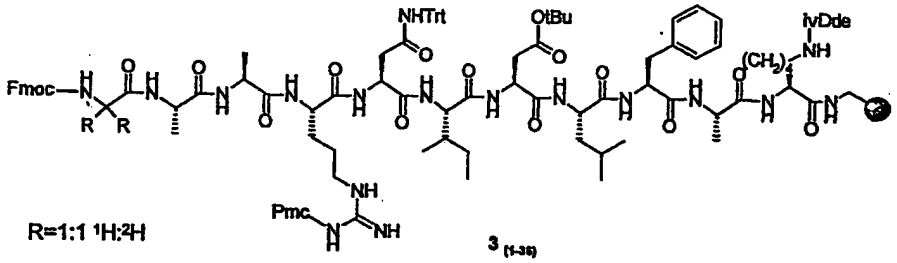
2(1)	Fmoc-Ala	Ala	Arg (Pmc)	Asn (Trt)	Ile	Asp (tBu)	Leu	Phe	Ala	Lys(ivDde)-Rink Resin
2(2)	Fmoc-Ala	Gly	Arg (Pmc)	Ala	Asn (Trt)	Ile	Asp (tBu)	Phe	Ala	Lys(ivDde)-Rink Resin
2(3)	Fmoc-Ala	Leu	Arg (Pmc)	Phe	Ala	Asn (Trt)	Ile	Phe	Ala	Lys(ivDde)-Rink Resin
2(4)	Fmoc-Ala	Phe	Arg (Pmc)	Phe	Ala	Ala	Asn (Trt)	Ile	Ala	Lys(ivDde)-Rink Resin
2(5)	Fmoc-Ala	Lys (Boc)	Arg (Pmc)	Ala	Gly	Ala	Ala	Phe	Ala	Lys(ivDde)-Rink Resin
2(6)	Fmoc-Ala	Gln (Trt)	Arg (Pmc)	Lys (Boc)	Val	Gly	Ala	Phe	Ala	Lys(ivDde)-Rink Resin
2(7)	Fmoc-Ala	Pro	Arg (Pmc)	Ser (OtBu)	Asp (tBu)	Val	Gly	Phe	Ala	Lys(ivDde)-Rink Resin
2(8)	Fmoc-Ala	Pro	Arg (Pmc)	Phe	Ser	Asp (tBu)	Val	Leu	Ala	Lys(ivDde)-Rink Resin
2(9)	Fmoc-Ala	Pro	Lys (Boc)	Ile	Gly	Ser (OtBu)	Asp (tBu)	Phe	Ala	Lys(ivDde)-Rink Resin
2(10)	Fmoc-Ala	Phe	Lys (Boc)	Ile	Ile	Gly	Ser (OtBu)	Phe	Ala	Lys(ivDde)-Rink Resin

2(11)	Fmoc-Ala	Arg(Pmc)	Lys (Boc)	Phe	Ile	Ile	Ile	Phe	Ala	Lys(ivDde)-Rink Resin
2(12)	Fmoc-Ala	Asp(tBu)	Lys (Boc)	Ile	Phe	Ser (OtBu)	Gly	Phe	Ala	Lys(ivDde)-Rink Resin
2(13)	Fmoc-Ala	Phe	Leu	Ala	Arg (Pmc)	Asn (Trt)	Ile	Asp (tBu)	Leu	Lys(ivDde)-Rink Resin
2(14)	Fmoc-Ala	Phe	Glu	Gly	Arg (Pmc)	Ala	Asn (Trt)	Ile	Asp (tBu)	Lys(ivDde)-Rink Resin
2(15)	Fmoc-Ala	Ser (OtBu)	Val	Leu	Arg (Pmc)	Phe	Ala	Asn (Trt)	Ile	Lys(ivDde)-Rink Resin
2(16)	Fmoc-Ala	Ala	Pro	Phe	Arg (Pmc)	Phe	Ala	Ala	Asn (Trt)	Lys(ivDde)-Rink Resin
2(17)	Fmoc-Ala	Ala	Phe	Lys (Boc)	Arg (Pmc)	Ala	Gly	Ala	Ala	Lys(ivDde)-Rink Resin
2(18)	Fmoc-Ala	Phe	Lys (Boc)	Gln	Arg (Pmc)	Lys	Val	Gly	Ala	Lys(ivDde)-Rink Resin
2(19)	Fmoc-Ala	Asp (tBu)	Ala	Pro	Arg (Pmc)	Ser (OtBu)	Asp (tBu)	Val	Gly	Lys(ivDde)-Rink Resin
2(20)	Fmoc-Ala	Phe	Gly	Pro	Arg (Pmc)	Phe	Ser (OtBu)	Asp	Val	Lys(ivDde)-Rink Resin
2(21)	Fmoc-Ala	Phe	Ala	Pro	Lys (Boc)	Ile	Gly	Ser (OtBu)	Asp (tBu)	Lys(ivDde)-Rink Resin
2(22)	Fmoc-Ala	Ala	Pro	Phe	Lys (Boc)	Ile	Ile	Gly	Ser (OtBu)	Lys(ivDde)-Rink Resin
2(23)	Fmoc-Ala	Gly	Val	Arg (Pmc)	Lys (Boc)	Phe	Ile	Ile	Gly	Lys(ivDde)-Rink Resin
2(24)	Fmoc-Ala	Asp (tBu)	Asp (tBu)	Asp (tBu)	Lys (Boc)	Ile	Phe	Ser (OtBu)	Ile	Lys(ivDde)-Rink Resin
2(25)	Fmoc-Ala	Leu	Phe	Gly	Phe	Leu	Ala	Arg (Pmc)	Asn (Trt)	Lys(ivDde)-Rink Resin
2(26)	Fmoc-Ala	Asp (tBu)	Phe	Leu	Phe	Glu	Gly	Arg (Pmc)	Ala	Lys(ivDde)-Rink Resin
2(27)	Fmoc-Ala	Ile	Phe	Asp (tBu)	Ser (OtBu)	Val	Leu	Arg (Pmc)	Phe	Lys(ivDde)-Rink Resin
2(28)	Fmoc-Ala	Asn (Trt)	Ile	Leu	Ala	Pro	Phe	Arg (Pmc)	Phe	Lys(ivDde)-Rink Resin
2(29)	Fmoc-Ala	Ala	Phe	Gly	Ala	Phe	Lys (Boc)	Arg (Pmc)	Ala	Lys(ivDde)-Rink Resin
2(30)	Fmoc-Ala	Ala	Phe	Leu	Phe	Lys (Boc)	Gln (Trt)	Arg (Pmc)	Lys	Lys(ivDde)-Rink Resin
2(31)	Fmoc-Ala	Gly	Phe	Phe	Asp (tBu)	Ala	Pro	Arg (Pmc)	Phe	Lys(ivDde)-Rink Resin
2(32)	Fmoc-Ala	Val	Leu	Asp (tBu)	Phe	Gly	Pro	Arg (Pmc)	Phe	Lys(ivDde)-Rink Resin
2(33)	Fmoc-Ala	Asp (tBu)	Phe	Leu	Phe	Ala	Pro	Lys (Boc)	Phe	Lys(ivDde)-Rink Resin
2(34)	Fmoc-Ala	Ser	Phe	Asp(tBu)	Ala	Pro	Phe	Lys(Boc)	Ile	Lys(ivDde)-Rink Resin
2(35)	Fmoc-Ala	Gly	Phe	Phe	Gly	Val	Arg(Pmc)	Lys(Boc)	Phe	Lys(ivDde)-Rink Resin
2(36)	Fmoc-Ala	Ile	Phe	Asp (tBu)	Asp (tBu)	Asp (tBu)	Asp (tBu)	Lys (Boc)	Ile	Lys(ivDde)-Rink Resin

Compounds $3_{(1-36)}$:

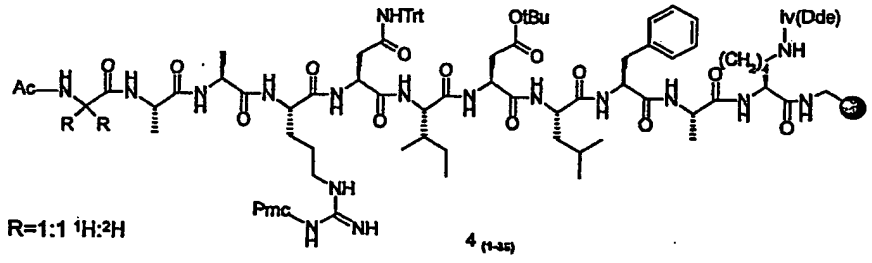
The Fmoc protected peptide resins $2_{(1-36)}$ (0.06 mmol) were transferred into 4 ml Alltech tubes and fitted in two batches onto a 24-position SPE Vacuum tank[3]. Each resin

sample was treated with a 20% Piperidine solution in DMF (2 ml) for (2 x10 min) and then washed with DMF (6 x 2 ml). The freshly deprotected resins were then treated with Fmoc-Gly-OH (0.027 g, 0.09 mmol) and Fmoc-(D2)Gly-OH (0.027 g, 0.09 mmol), PyBOP (0.094 g, 0.18 mmol), and HOBt (0.024 g, 0.18 mmol) in a mixture of DMF (1 ml) and DCM (0.5 ml). Finally, Hünig's base (0.047 g, 0.36 mmol) was added and the reactions allowed to proceed at room temperature for 16 h with intermittent manual agitation. The resins were then washed successively with DMF (6 x 2 ml) and dichloromethane (4 x 2 ml) to give ***compounds 3(1-36)*** (ca 0.350 g, 0.06 mmol) as yellow solids. Kaiser test was negative in each case.



Compounds $4_{(1-36)}$:

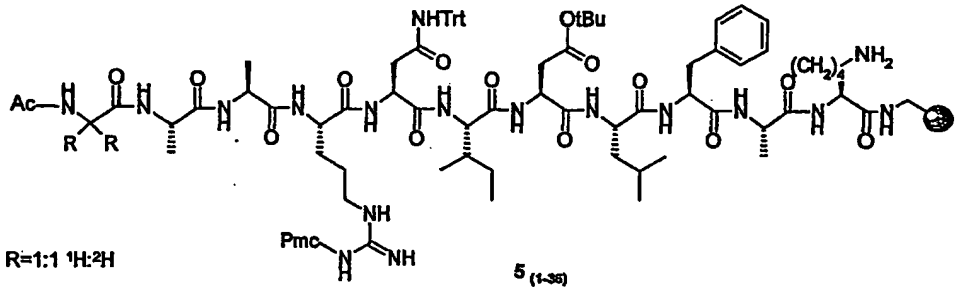
The Fmoc protected peptide resins $3_{(1-36)}$ (0.06 mmol) were treated with a 20% Piperidine solution in DMF (2 ml) for (2 x 10 min) and then washed with DMF (6 x 2 ml). A 20% solution of acetic anhydride in DMF (2 ml) was then added to each resin sample and the reaction allowed to proceed for 4 h whilst resin samples were manually agitated intermittently. The resins were then washed successively with DMF (6 x 2 ml) and dichloromethane (4 x 2 ml) to give ***compounds $4_{(1-36)}$*** (ca 0.310 g, 0.06 mmol) as yellow solids. Kaiser test was negative in each case.



Compounds $5_{(1-36)}$:

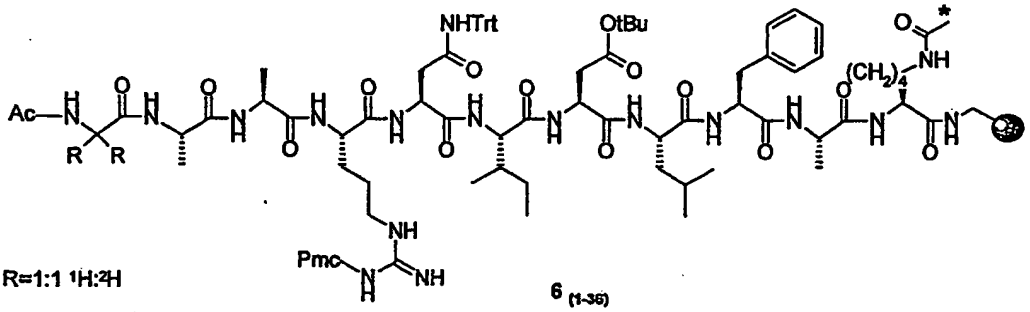
The iv(Dde) protected peptide resins $4_{(1-36)}$ (0.06 mmol) were treated with a 20% solution of Hydrazine in DMF (2 ml) for (2 x 20 min). The resins were then washed

successively with DMF (6 x 2 ml) and dichloromethane (4 x 2 ml) to give ***amine resins*** $5_{(1\text{-}36)}$ (ca 0.300 g, 0.06 mmol) as yellow solids. Kaiser test was positive in all cases.



Compounds $6_{(1\text{-}36)}$ *:*

The amine resins $5_{(1\text{-}36)}$ (0.06 mmol) were treated with a solution of Acetic anhydride (0.2 ml) and Acetic anhydride-d_6 (0.2 ml) in DMF (1.8 ml) and the reaction was allowed to proceed for 4 h with intermittent manual shaking. The resins were then washed successively with DMF (6 x 2 ml), dichloromethane (4 x 2 ml) and ether (2 x 2 ml) then dried *in vacuo* to give ***peptide resins*** $6_{(1\text{-}36)}$ (ca 0.400 g, 0.06 mmol) as yellow solids. Kaiser test was negative in each case.



Compounds $7_{(1\text{-}36)}$ *:*

The peptide resins $6_{(1\text{-}36)}$ (0.06 mmol) were swollen in dichloromethane for 10 min and the solvents drained under vacuum. Trifluoroacetic acid (1.5 ml) was added to each resin sample and the cleavage reaction allowed to proceed for 2 h. The resins were drained and then further washed with dichloromethane (6 x 1 ml). The combined filtrates were concentrated under a stream of nitrogen then dried *in vacuo* to give ***DiMaS tagged peptides*** $7_{(1\text{-}36)}$ (ca 0.5 mg) as white solids or pale brown oils. The expected multiplet pattern was observed in all 36 cases (Table 3) MS Analysis (Figure 12).

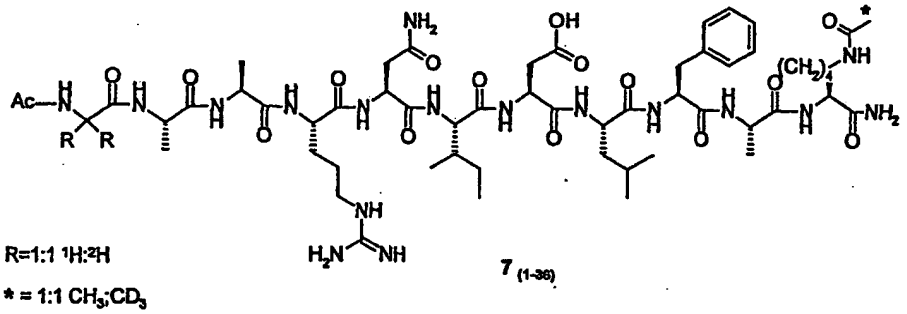


Table 3: Characterisation of Compounds $7_{(1-36)}$:

no												Av Mw
7(1)	Ac-Gly*	Ala	Ala	Arg	Asn	Ile	Asp	Leu	Phe	Ala	Lys(Ac*)-NH2	1259
7(2)	Ac-Gly*	Ala	Gly	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1203
7(3)	Ac-Gly*	Ala	Leu	Arg	Phe	Ala	Asn	Ile	Phe	Ala	Lys(Ac*)-NH2	1291
7(4)	Ac-Gly*	Ala	Phe	Arg	Phe	Ala	Ala	Asn	Ile	Ala	Lys(Ac*)-NH2	1249
7(5)	Ac-Gly*	Ala	Lys	Arg	Ala	Gly	Ala	Ala	Phe	Ala	Lys(Ac*)-NH2	1131
7(6)	Ac-Gly*	Ala	Gln	Arg	Lys	Val	Gly	Ala	Phe	Ala	Lys(Ac*)-NH2	1216
7(7)	Ac-Gly*	Ala	Pro	Arg	Ser	Asp	Val	Gly	Phe	Ala	Lys(Ac*)-NH2	1188
7(8)	Ac-Gly*	Ala	Pro	Arg	Phe	Ser	Asp	Val	Leu	Ala	Lys(Ac*)-NH2	1244
7(9)	Ac-Gly*	Ala	Pro	Lys	Ile	Gly	Ser	Asp	Phe	Ala	Lys(Ac*)-NH2	1174
7(10)	Ac-Gly*	Ala	Phe	Lys	Ile	Ile	Gly	Ser	Phe	Ala	Lys(Ac*)-NH2	1222
7(11)	Ac-Gly*	Ala	Arg	Lys	Phe	Ile	Ile	Ile	Phe	Ala	Lys(Ac*)-NH2	1347
7(12)	Ac-Gly*	Ala	Asp	Lys	Ile	Phe	Ser	Gly	Phe	Ala	Lys(Ac*)-NH2	1224
7(13)	Ac-Gly*	Ala	Phe	Leu	Ala	Arg	Asn	Ile	Asp	Leu	Lys(Ac*)-NH2	1301
7(14)	Ac-Gly*	Ala	Phe	Glu	Gly	Arg	Ala	Asn	Ile	Asp	Lys(Ac*)-NH2	1261
7(15)	Ac-Gly*	Ala	Ser	Val	Leu	Arg	Phe	Ala	Asn	Ile	Lys(Ac*)-NH2	1259
7(16)	Ac-Gly*	Ala	Ala	Pro	Phe	Arg	Phe	Ala	Ala	Asn	Lys(Ac*)-NH2	1233
7(17)	Ac-Gly*	Ala	Ala	Phe	Lys	Arg	Ala	Gly	Ala	Ala	Lys(Ac*)-NH2	1131
7(18)	Ac-Gly*	Ala	Phe	Lys	Gln	Arg	Lys	Val	Gly	Ala	Lys(Ac*)-NH2	1273
7(19)	Ac-Gly*	Ala	Asp	Ala	Pro	Arg	Ser	Asp	Val	Gly	Lys(Ac*)-NH2	1156
7(20)	Ac-Gly*	Ala	Phe	Gly	Pro	Arg	Phe	Ser	Asp	Val	Lys(Ac*)-NH2	1264
7(21)	Ac-Gly*	Ala	Phe	Ala	Pro	Lys	Ile	Gly	Ser	Asp	Lys(Ac*)-NH2	1174
7(22)	Ac-Gly*	Ala	Ala	Pro	Phe	Lys	Ile	Ile	Gly	Ser	Lys(Ac*)-NH2	1172
7(23)	Ac-Gly*	Ala	Gly	Val	Arg	Lys	Phe	Ile	Ile	Gly	Lys(Ac*)-NH2	1229
7(24)	Ac-Gly*	Ala	Asp	Asp	Asp	Lys	Ile	Phe	Ser	Ile	Lys(Ac*)-NH2	1292
7(25)	Ac-Gly*	Ala	Leu	Phe	Gly	Phe	Leu	Ala	Arg	Asn	Lys(Ac*)-NH2	1277
7(26)	Ac-Gly*	Ala	Asp	Phe	Leu	Phe	Glu	Gly	Arg	Ala	Lys(Ac*)-NH2	1294
7(27)	Ac-Gly*	Ala	Ile	Phe	Asp	Ser	Val	Leu	Arg	Phe	Lys(Ac*)-NH2	1336
7(28)	Ac-Gly*	Ala	Asn	Ile	Leu	Ala	Pro	Phe	Arg	Phe	Lys(Ac*)-NH2	1317
7(29)	Ac-Gly*	Ala	Ala	Phe	Gly	Ala	Phe	Lys	Arg	Ala	Lys(Ac*)-NH2	1217
7(30)	Ac-Gly*	Ala	Ala	Phe	Leu	Phe	Lys	Gln	Arg	Lys	Lys(Ac*)-NH2	1377
7(31)	Ac-Gly*	Ala	Gly	Phe	Phe	Asp	Ala	Pro	Arg	Phe	Lys(Ac*)-NH2	1296
7(32)	Ac-Gly*	Ala	Val	Leu	Asp	Phe	Gly	Pro	Arg	Phe	Lys(Ac*)-NH2	1290
7(33)	Ac-Gly*	Ala	Asp	Phe	Leu	Phe	Ala	Pro	Lys	Phe	Lys(Ac*)-NH2	1324
7(34)	Ac-Gly*	Ala	Ser	Phe	Asp	Ala	Pro	Phe	Lys	Ile	Lys(Ac*)-NH2	1264
7(35)	Ac-Gly*	Ala	Gly	Phe	Phe	Gly	Val	Arg	Lys	Phe	Lys(Ac*)-NH2	1297

Example 9: Incubation of Compounds 7(2) and 7(13) with Trypsin:

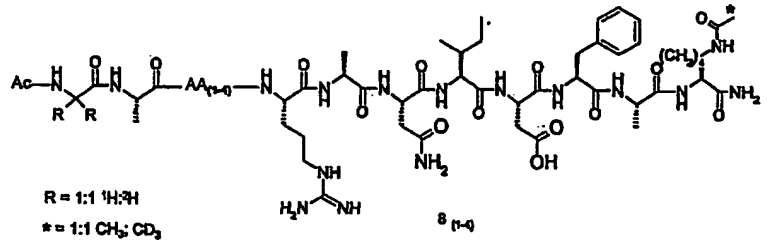
Compounds 7(2) and 7(13) (10 µl in Acetonitrile) were treated with a solution of buffer (50 mM HEPES, 150 mM NaCl and 1 µM Zn^{2+}, 35 µl), and finally Trypsin was added (5µl, 2.5 µM). The reaction was allowed to take place at room temperature for 9h and an aliquot of solution was removed for LC-MS analysis every 2 h.

Incubation of peptides 7(2) and 7(13) with Trypsin as described above gave the expected peptide fragments corresponding to cleavage after the arginine residue. Both sets of doublets were observed in most cases (MS Analysis shown in Figure 13).

Example 10: Preparation of Pools

Compounds 8 $_{(1-4)}$:

The linear peptide was assembled on an ABI 433A automated peptide synthesizer. The Fmoc protected Rink amide resin (0.12 g, 0.06 mmol) was coupled successively with Fmoc-Lys(ivDde)-OH, Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ala-OH and Fmoc-Arg(Pmc)-OH, using the HBTU (1.0 mmol) activation cycle and extended coupling time (2h). The introduction of a mixture of (Fmoc-Cha-OH : Fmoc-Pro-OH : Fmoc-Leu-OH : Fmoc-Glu-OH) (1:1:1:1), (1.00 mmol total) was performed online. This was followed by coupling with Fmoc-Ala-OH, (1.0 mmol), then coupled with a mixture of [Fmoc-(H_2)Gly-OH : Fmoc-(D_2)Gly-OH) (1:1), (1 mmol total). Finally the assembled peptide resin was transferred into an Alltech tube and was treated with a solution of Acetic anhydride (0.2 ml) and Acetic anhydride-d_6 (0.2 ml) in DMF (1.8 ml) and the reaction was allowed to proceed for 4 h with intermittent manual shaking. The resin was then washed successively with DMF (6 x 2 ml), dichloromethane (4 x 2 ml) and ether (2 x 2 ml) then dried *in vacuo* to give ***peptide resin 8$_{(1-4)}$*** (ca 0.400 g, 0.06 mmol) as a yellow solid. Kaiser test was negative.



AA1(1-4) = Cha : Pro : Leu : Glu
1: 1 :1 :1

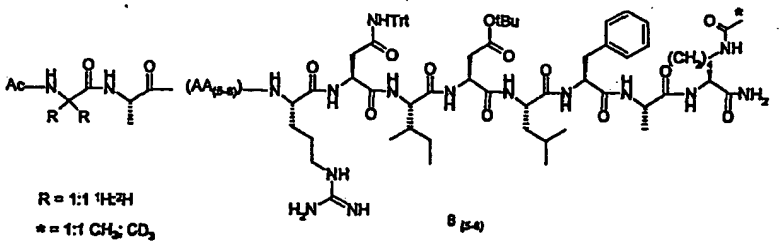
The expected multiplet pattern was observed in all four cases (Table 4) MS Analysis bj000051-1 (Figure 14).

Table 4: Characterisation of Peptides $8_{(1-4)}$:

											MW
8(1)	Ac-Gly*	Ala	Cha	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1299
8(2)	Ac-Gly*	Ala	Pro	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1243
8(3)	Ac-Gly*	Ala	Leu	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1259
8(4)	Ac-Gly*	Ala	Glu	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1275

Compounds $8_{(5-8)}$:

Peptides $8_{(5-8)}$ were prepared in an analogous fashion to peptides $8_{(1-4)}$. The expected MS pattern was observed in all four cases. The expected multiplet pattern was observed in all four cases (Table 5) MS Analysis bj000051-2 (Figure 15).



AA(5-8): Ala : VAl : Ser : His
1 : 1 : 1: 1

Table 5: Characterisation of Peptides $8_{(5-8)}$:

											MW
8(5)	Ac-Gly*	Ala	Ala	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1217
8(6)	Ac-Gly*	Ala	Val	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1245
8(7)	Ac-Gly*	Ala	Ser	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1233
8(8)	Ac-Gly*	Ala	His	Arg	Ala	Asn	Ile	Asp	Phe	Ala	Lys(Ac*)-NH2	1283

Example 11: Incubation with Trypsin

Incubation of Compounds $8_{(1-4)}$ with Trypsin

The peptide mixture $8_{(1-4)}$ (30 µl in Acetonitrile) was treated with a solution of buffer (50 mM HEPES, 150 mM NaCl and 1 µM Zn^{2+}, 100 µl), and finally Trypsin was added (10µl, 5 µM). The reaction was allowed to take place at room temperature for 9h and an aliquot of solution was removed for LC-MS analysis every 2 h. Incubation with Trypsin gave the expected peptide fragments corresponding to cleavage after the arginine residue. Both sets of doublets observed in most cases (Table 6) MS Analysis bj000051-5 (Figure 16).

Table 6: Results of incubation of Compounds $8_{(1-4)}$ with Trypsin:

	Mw	LHS	RHS
8(1)	1299	498, 500	819, 822
8(2)	1243	442, 444	819, 822
8(3)	1259	458, 460	819, 822
8(4)	1275		819, 822

Incubation of Compounds $8_{(5-8)}$ with Trypsin:

The peptide mixture $8_{(5-8)}$ was incubated with Trypsin as described above to give the expected peptide fragments corresponding to cleavage after the arginine residue. Both sets of doublets observed in most cases (Table 7) MS Analysis bj000051-6 (Figure 17).

Table 7: Results of incubation of Compounds $8_{(5-8)}$ with Trypsin

	Mw	LHS	RHS
8(5)	1217	416, 418	819, 822
8(6)	1245	444, 446	819, 822
8(7)	1233	432, 434	819, 822
8(8)	1283	(—)	819, 822

Example 12: Peptide Sequencing:

The sequence or primary structure of a peptide may be determined by cleaving or fragmentation of the peptide and subsequent analysis by mass spectrometry.

In the case of a peptide which has been differentially isotopically labelled at the N-terminus amino acid, fragmentation in the C- to N- direction retains the peak split caused by the isotopic label giving rise to doublets, while the reverse fragmentation in the N- to C- direction produces only single mass peaks (Figure 18).

This data can then be used to determine the amino acid sequence of the peptide. The difference in sizes of the fragments will indicate the sizes of the amino acids which have been removed, and this data may be used to determine the primary sequence of the original molecule.

References:

1. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.*, **1970**, 34, 595.
2. Atherton, E.; Sheppard, R. C. In '*Solid Phase Peptide Synthesis*', **1989**, Practical Approach Series, IRL Press, Oxford.
3. Available from Whatman

Abbreviations

AcOH = Acetic Acid

Boc = *tert*-Butyloxycarbonyl

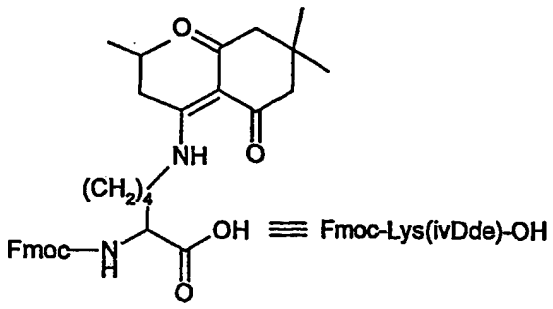
DIPEA = Diispropylethylamine

DMAP = Dimethylaminopyridine

DMF = Dimethylformamide

Fmoc = Fluorenylmethoxycarbonyl

Fmoc-Lys(ivDde)-OH = N-α-Fmoc-N-ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-L-lysine.



≡ Fmoc-Lys(ivDde)-OH

HBTU = 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt = Hydroxybenzotriazole

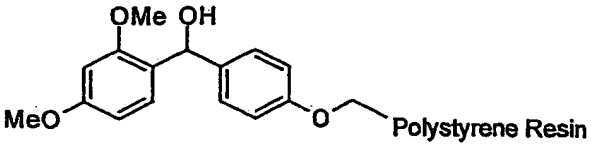
NMP = *N*-Methyl pyrrolidone

MS = Mass Spectroscopy

PG = Protecting group

PyBOP = Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate

Rink Amide Resin = 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)



TFA = Trifluoroacetic Acid

Bzl = Benzyl

Cbz = Carbobenzyloxy

DCM = Dichloromethane

Fmoc = Fluorenylmethoxycarbonyl

TFA = Trifluroacetic Acid

TIPS = Triisopropylsilane

CLAIMS

1. A method of analysing cleavage of a polymer, the method comprising:

(a) providing a sample of said polymer, a portion of said polymer molecules having been labelled at a position on one side of a potential cleavage site with a first isotopic label and a portion of said polymer molecules having been labelled at a position on the opposite side of the potential cleavage site with a second isotopic label;

(b) incubating said sample under conditions suitable for cleavage at said potential cleavage site, and

(c) analysing the mass(es) of any cleaved fragments by mass spectrometry and thereby determining whether and/or where cleavage has taken place.

2. A method according to claim 1 wherein the mass change caused by the presence of the first isotopic label compared with the mass in the presence of the normal element(s) at that position is different to the mass change caused by the presence of the second isotopic label compared with the mass in the presence of the normal element(s) at that position.

3. A method according to claim 1or 2 wherein

(a) 50% of the polymer molecules in said sample comprise said first isotopic label, and/or

(b) 50% of the polymer molecules in said sample comprise said second isotopic label.

4. A method according to any one of claims 1 to 3 wherein said sample comprises a portion of polymer molecules which comprise both said first isotopic label and said second isotopic label, a portion of polymer molecules which comprise said first isotopic label but not said second isotopic label, a portion of polymer molecules which comprise said second isotopic label but not said first isotopic label and a portion of said polymer labels which comprise neither said first nor second isotopic labels.

5. A method of screening a library of polymers for cleavage, the method comprising:

(a) providing a library comprising two or more polymers labelled as described in any one of the preceding claims,

(b) incubating said library of polymers under conditions suitable for cleavage, and

(c) analysing the mass(es) of any cleaved fragments by mass spectrometry and thereby determining whether and/or where cleavage has taken place.

6. A method according to any one of the preceding claims wherein the first and second isotopic labels comprise different chemical isotopes.

7. A method according to any one of claims 1 to 5 wherein the first and second isotopic labels comprise the same chemical isotope and wherein each of said first and second istopic labels comprise a different number of atoms of said chemical isotope.

8. A method according to any one of the preceding claims wherein said isotopic labels comprise ^{2}H, ^{13}C, ^{18}O, ^{15}N and/or ^{81}Br.

9. A method according to any one of the preceding claims wherein said polymer is a linear polymer.

10. A method according to any one of the preceding claims wherein said polymer comprises a peptide or protein.

11. A method according to claim 10 wherein said first and second isotopic labels are located in the terminal amino acids of the peptide or protein.

12. A method according to any one of the preceding claims wherein said polymer further comprises a moeity which allows for separation of the molecule attached to the moeity from other material.

13. A method according to any one of the preceding claims wherein the sample or library is incubated with an enzyme under conditions suitable for cleavage of a substrate.

14. A method according to claim 13 wherein said enzyme is a protease.

15. A method according to any one of the preceding claims wherein the mass(es) of the cleaved fragments are used to calculate the location of the cleavage site.

16. A method according to any one of the preceding claims, the method further comprising the step of quantifying the amount of the polymer or cleaved fragment(s) present after cleavage.

17. A method according to any one of the preceding claims wherein said polymer further comprises a detectable non-isotopic label on one side of the potential cleavage site and an inhibitor for said label on the opposite side of the potential cleavage site, the method comprising the additional step of detecting said detectable non-isotopic label, wherein the detection of said label indicates that said polymer has been cleaved.

18. A method according to claim 17 wherein said detectable label is a fluorescent group and said inhibitor is a quencher for said fluorescent group.

19. A method according to any one of the preceding claims wherein said polymer is attached to a solid support.

20 A method according to claim 19 wherein the polymer is attached via a releasable linker molecule.

21. A method according to claim 19 or 20 wherein said solid support is a resin bead.

22. A method according to any one of the preceding claims further comprising determining the structure or sequence of one or both of the cleaved fragments.

23. A method according to claim 22 wherein the cleaved fragment(s) are further cleaved and the masses of these further fragments determined using mass spectrometry.

24. A kit for screening a library of polymers for cleavage, comprising two or more polymers as described in any one of claims 1 to 12 or 17 to 21.

25. A kit according to claim 24 wherein said polymers are supplied in solid form or in solutions.

26. A kit according to claim 24 wherein said polymers are supplied on a solid support as described in any one of claims 19 to 21.

27. A kit according to claim 26 further comprising a linker cleaving reagent.

28. A kit for the preparation of a polymer as described in claim 1, comprising a set of chemical monomers which comprises monomers which have been isotopically labelled and monomers which have no isotopic label, in a form suitable for polymer synthesis.

29. A kit according to claim 28 further comprising means for adding an isotopic label to an unlabelled monomer.

30. A kit according to claim 28 or 29 wherein said set of monomers comprises:

(a) a number of isotopically labelled amino acids and a number of amino acids which have no isotopic label; or

(b) a number of isotopically labelled nucleotides and a number of nucleotides which have no isotopic label.

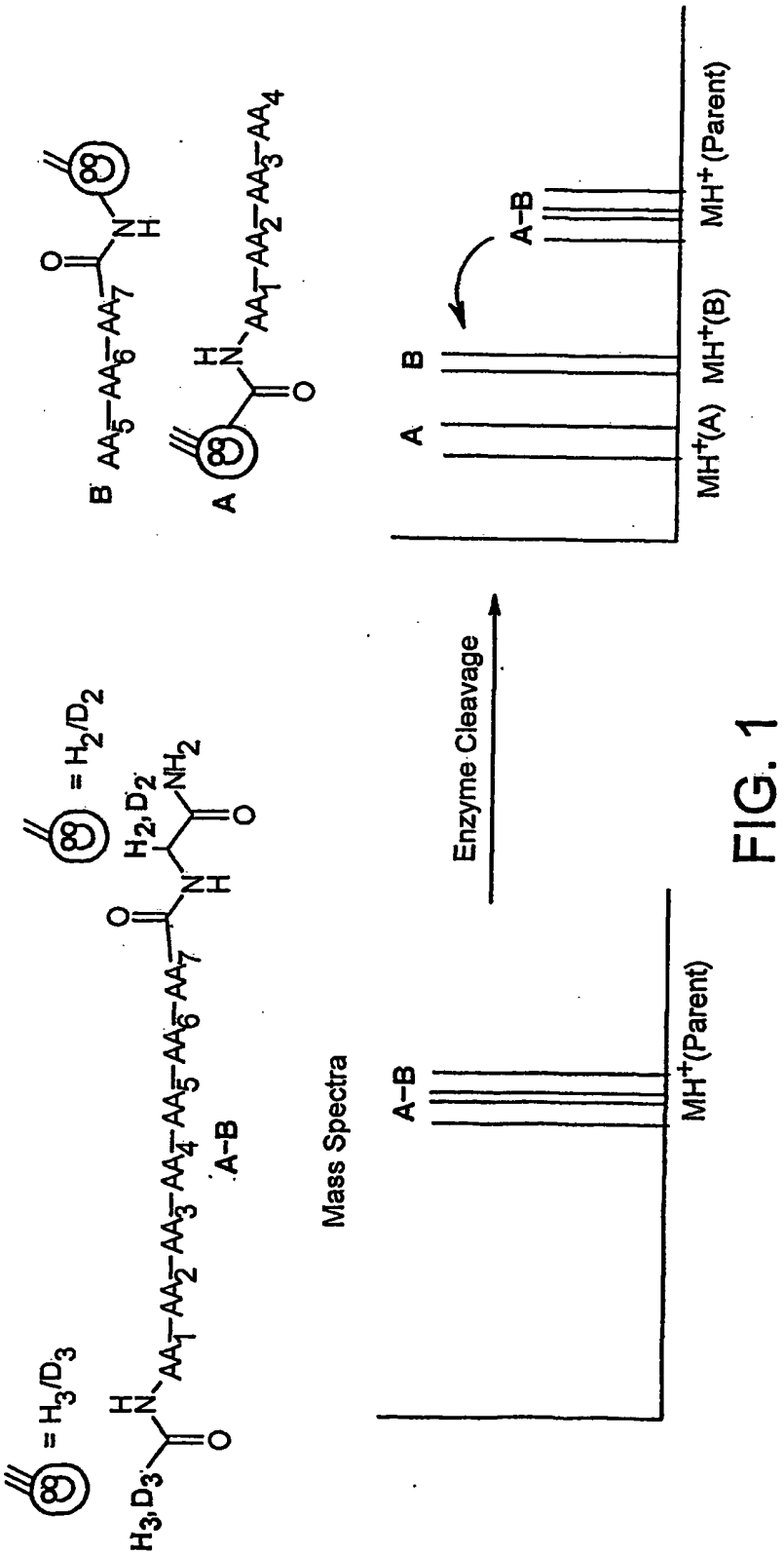

= H_3/D_3
H_3,D_3
AA_1–AA_2–AA_3–AA_4–AA_5–AA_6–AA_7
A-B
= H_2/D_2
H_2,D_2
Mass Spectra
A-B
MH^+(Parent)
Enzyme Cleavage
B
AA_5–AA_6–AA_7
A
AA_1–AA_2–AA_3–AA_4
A
B
A-B
MH^+(A)
MH^+(B)
MH^+(Parent)

FIG. 1

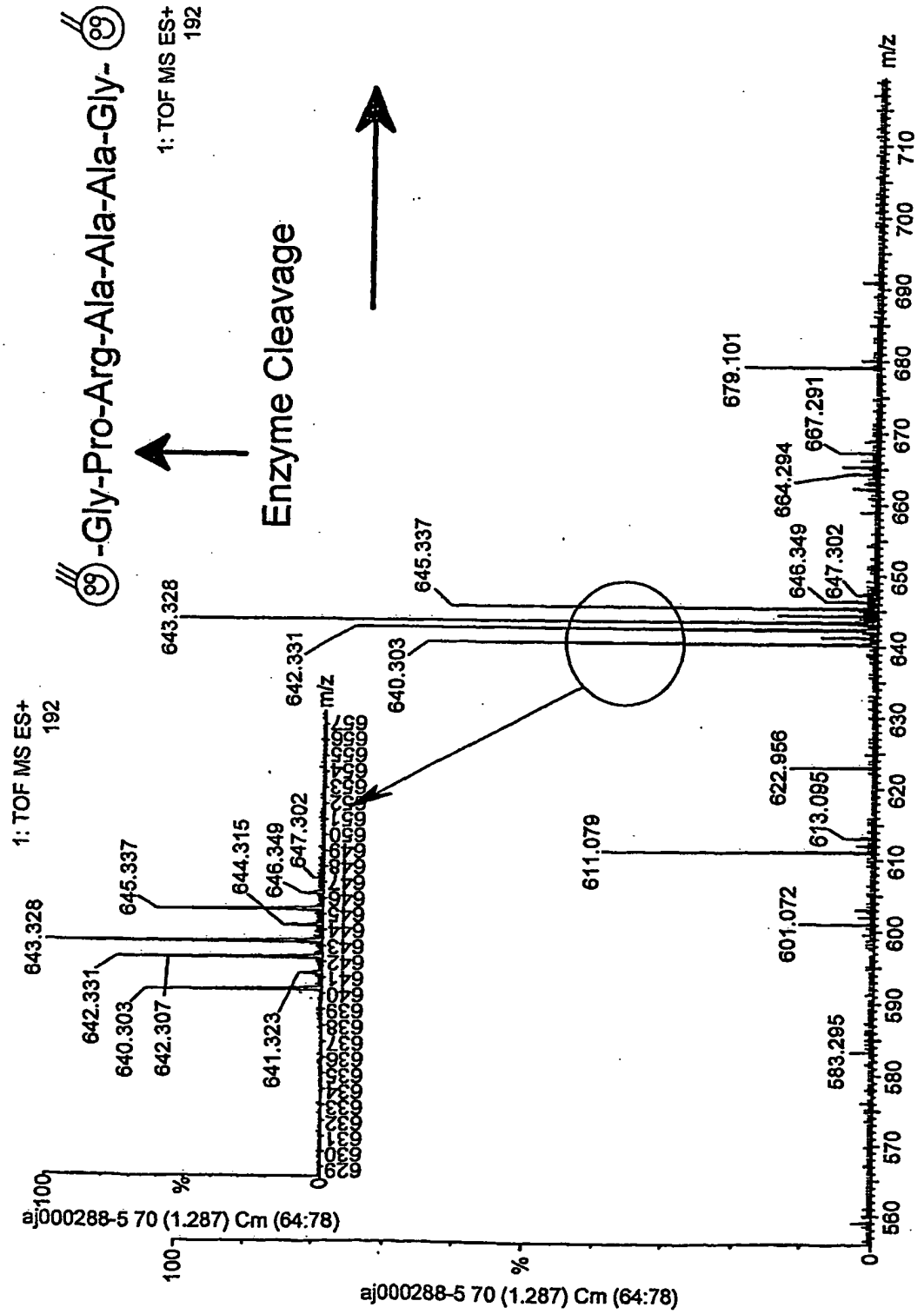
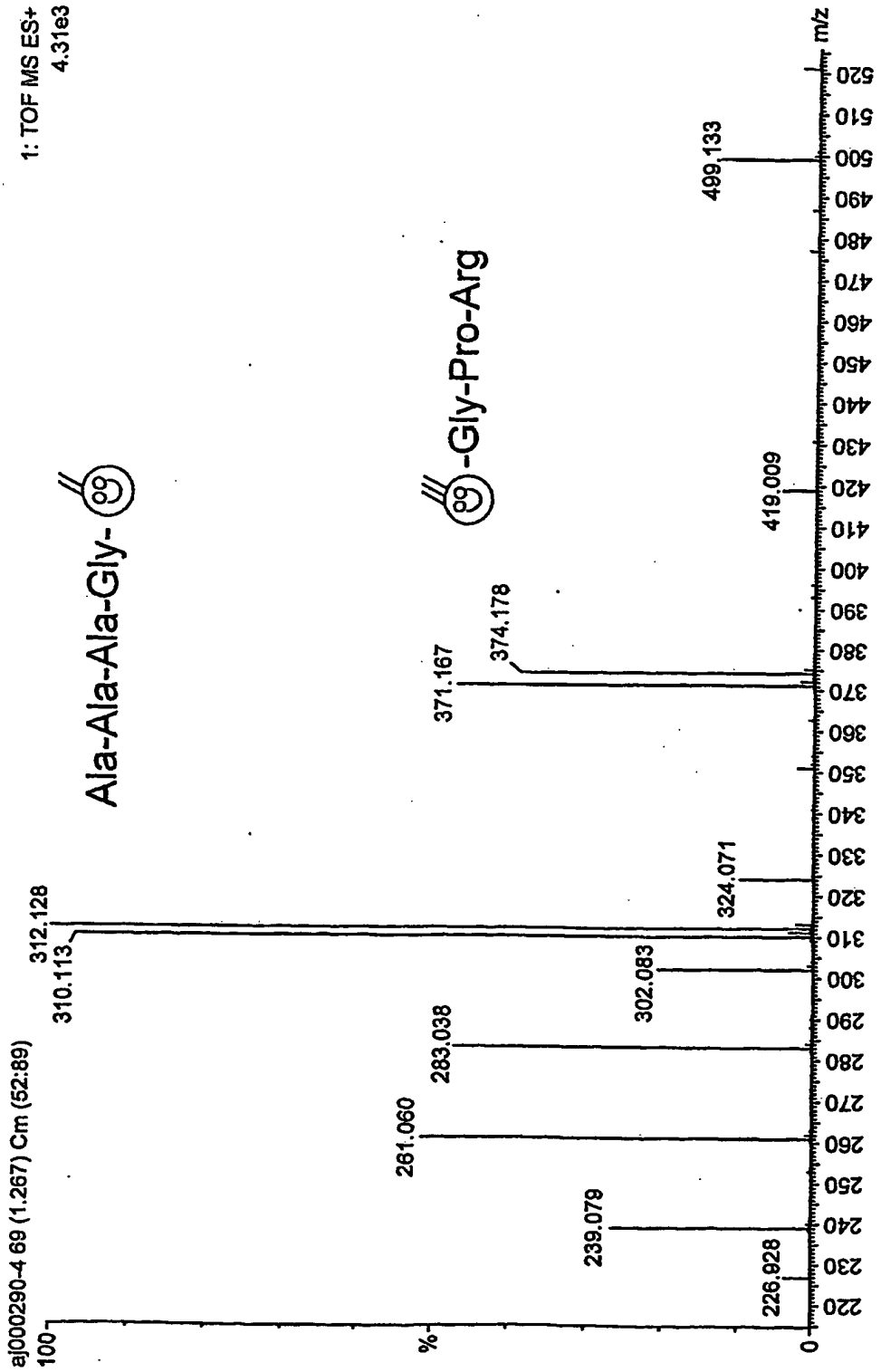


FIG. 2

BEST AVAILABLE COPY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100
%
0
m/z
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-Gly-Pro-Arg
312.128
310.113
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302.083
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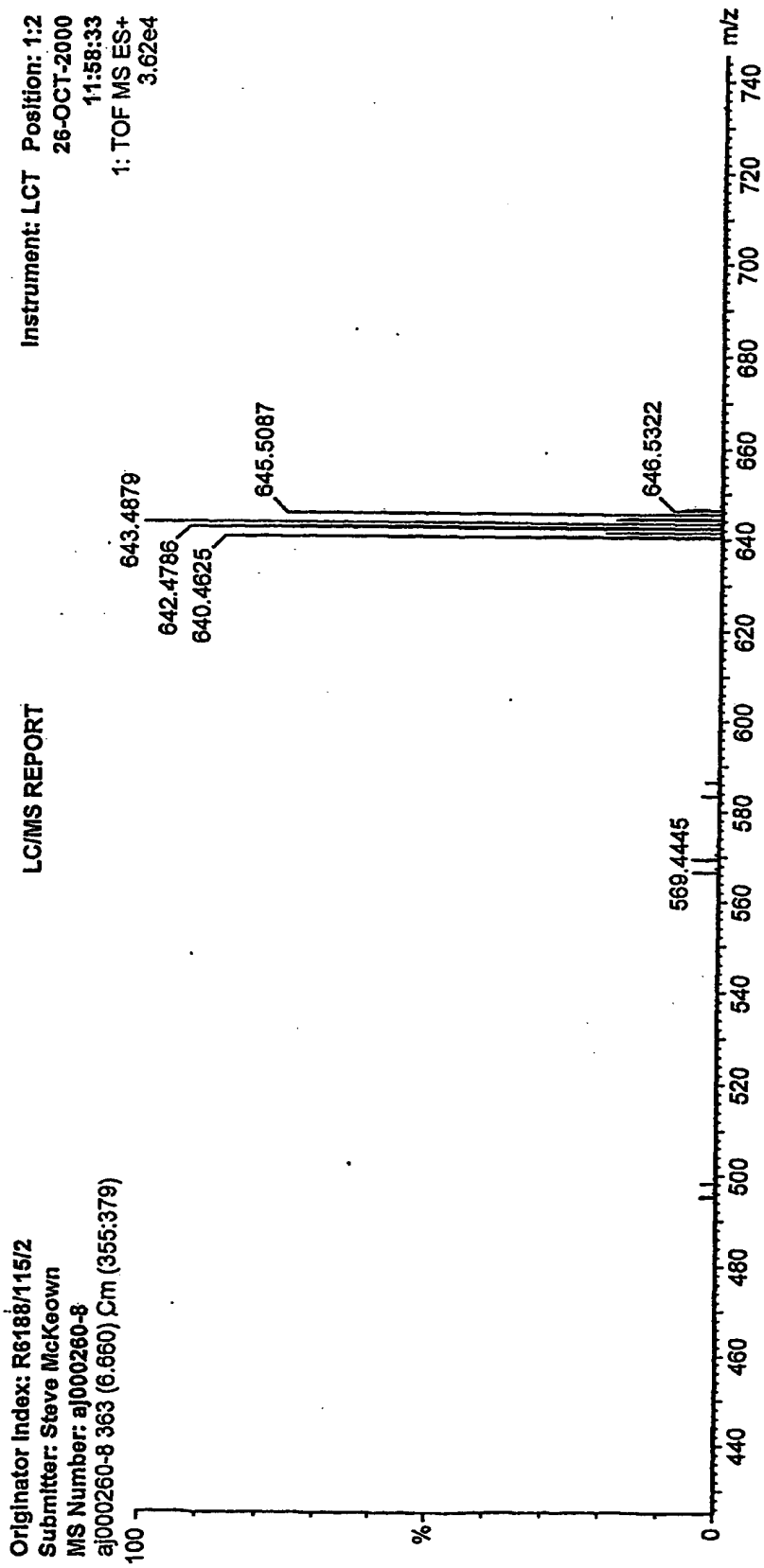
261.060
239.079
226.928
371.167
374.178
419.009
499.133

FIG. 2 CONT'D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569.4445
100
%
0
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580
600
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660
680
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720
740
m/z

FIG. 3

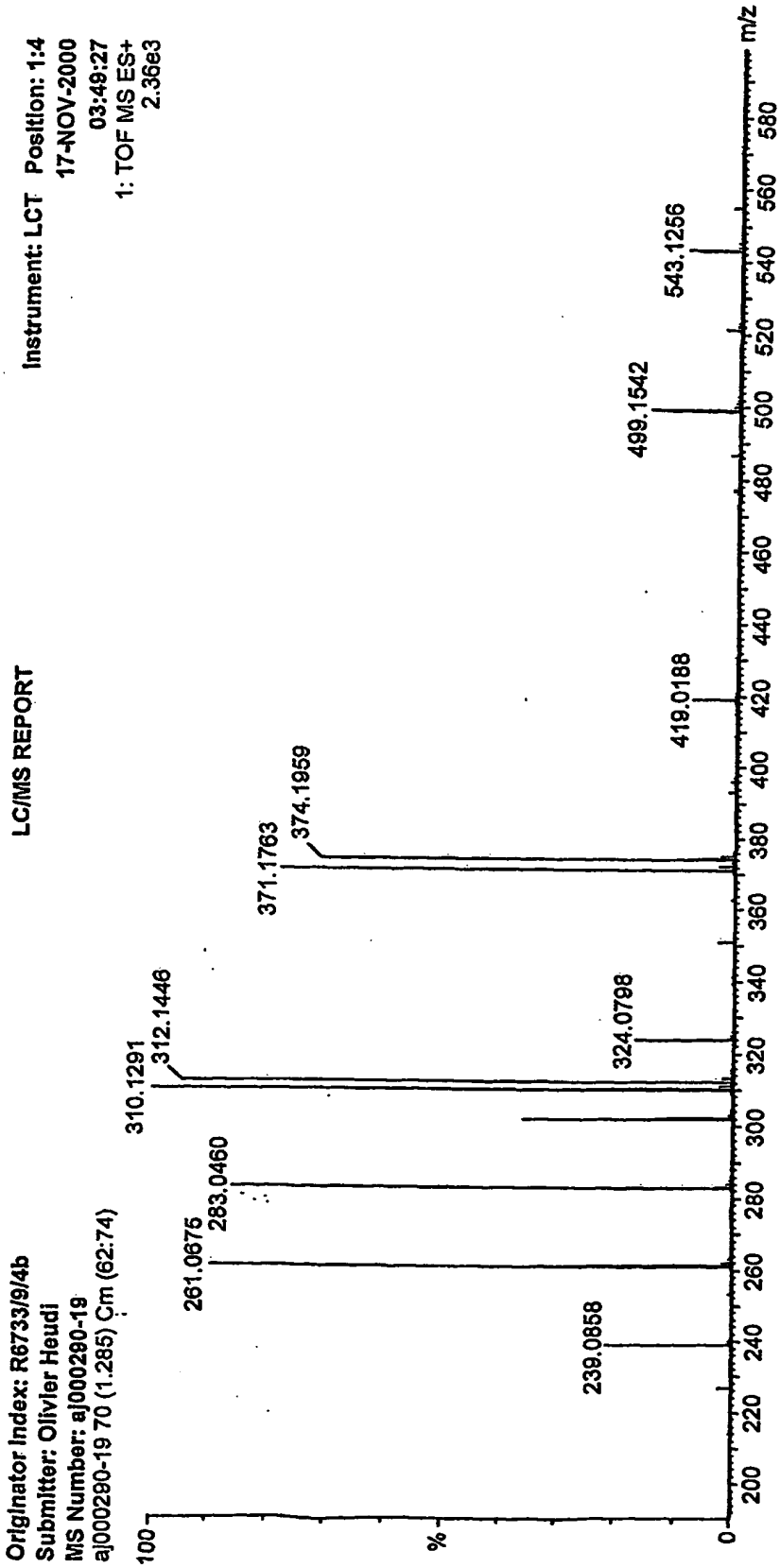
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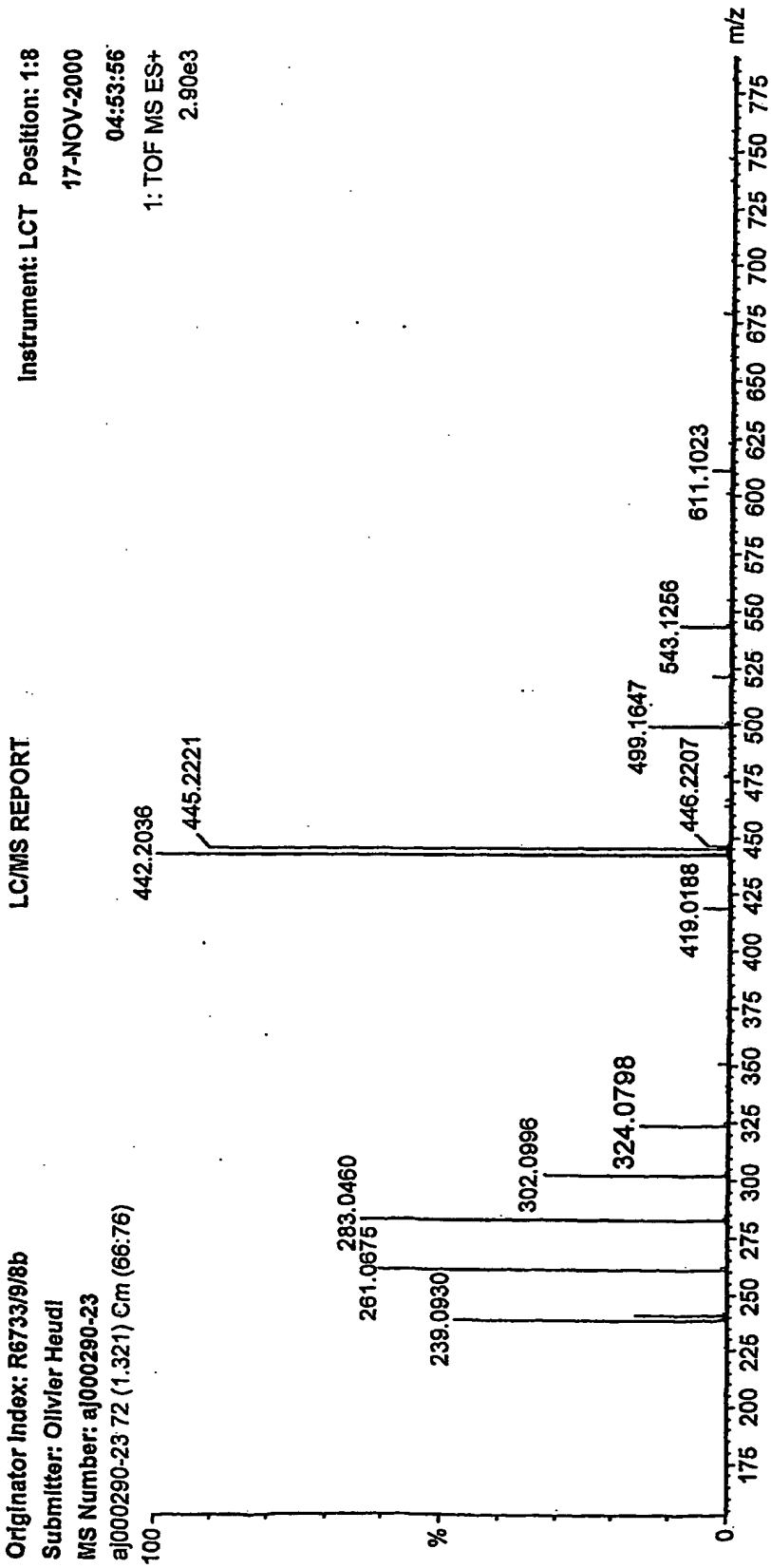
FIG. 4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m/z
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283.0460
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324.0798
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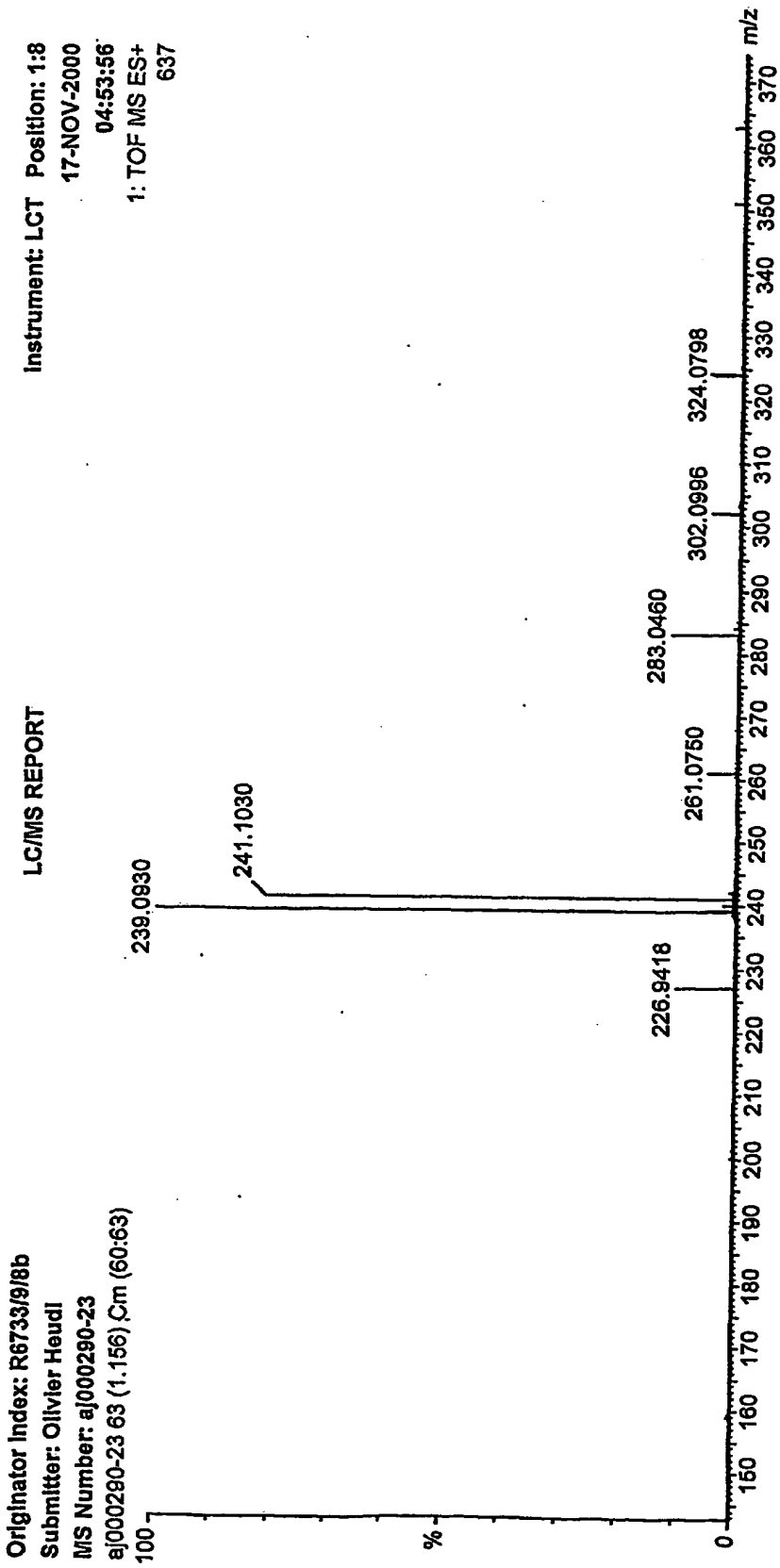
611.1023

FIG. 5a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226.9418
261.0750
283.0460
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324.0798
100
%
0
150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 m/z

FIG. 5b

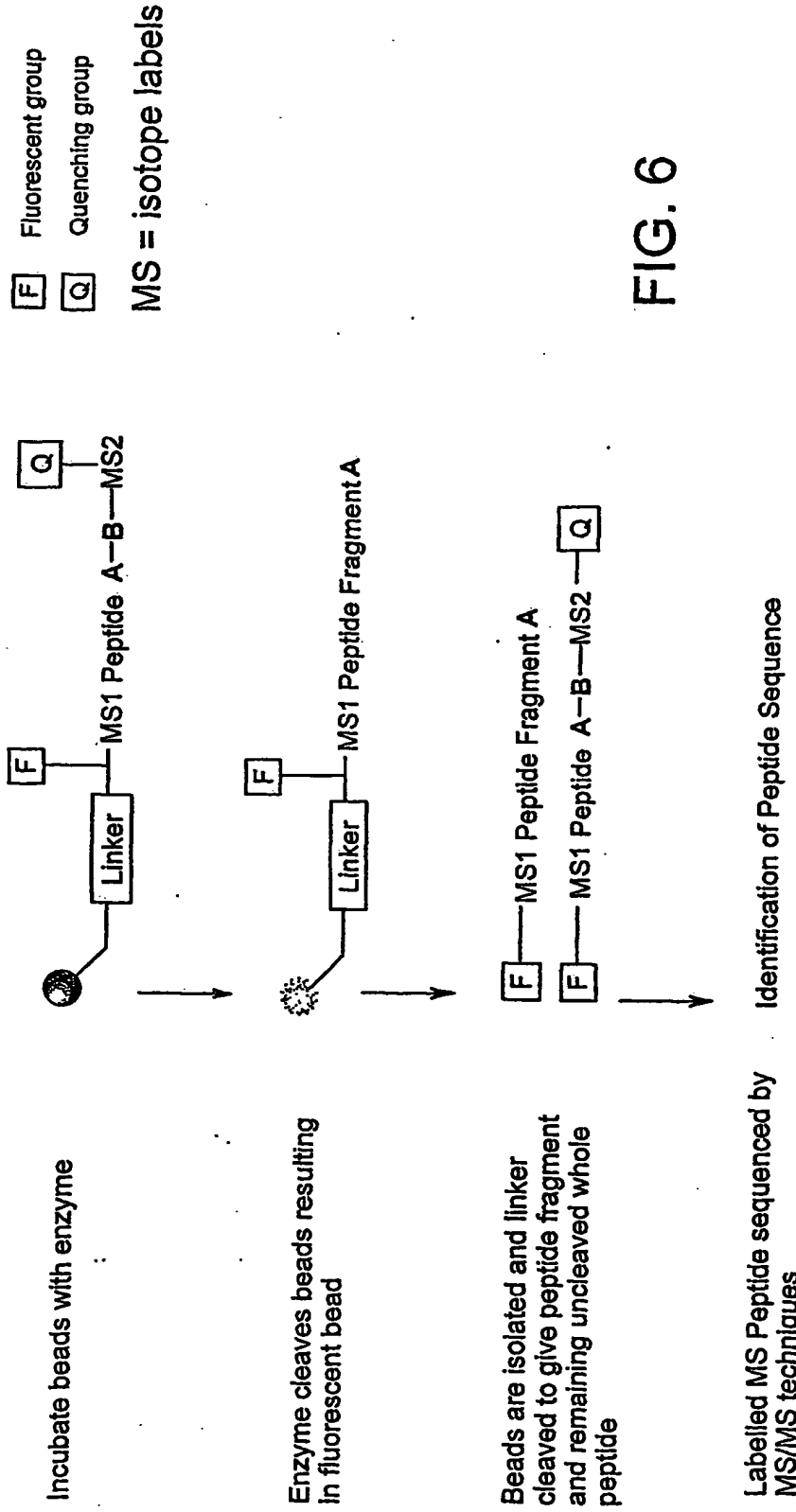

F Fluorescent group
Q Quenching group
MS = isotope labels
Incubate beads with enzyme
Linker
MS1 Peptide A—B—MS2
Enzyme cleaves beads resulting in fluorescent bead
MS1 Peptide Fragment A
Beads are isolated and linker cleaved to give peptide fragment and remaining uncleaved whole peptide
MS1 Peptide Fragment A
MS1 Peptide A—B—MS2
Labelled MS Peptide sequenced by MS/MS techniques
Identification of Peptide Sequence

FIG. 6

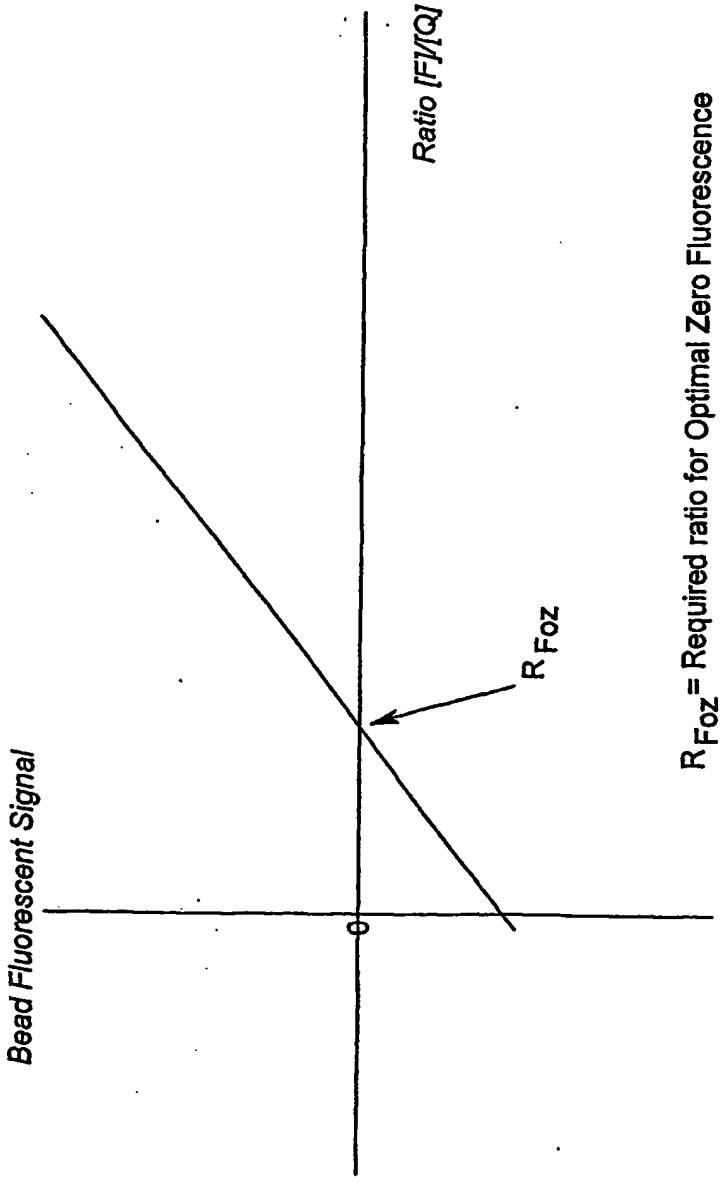
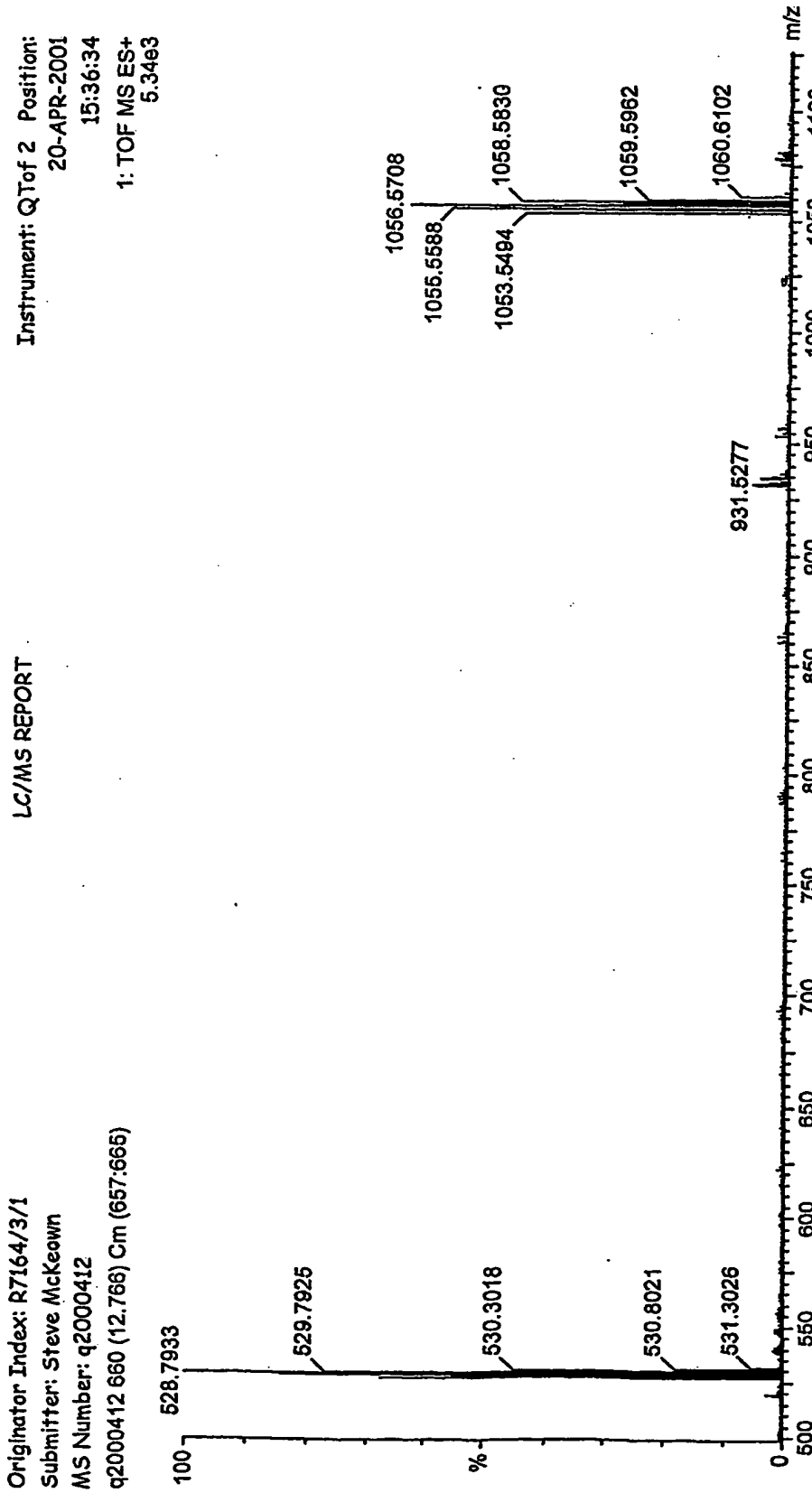

Ratio [F]/[Q]
Bead Fluorescent Signal
0
R_{Foz}
R_{Foz} = Required ratio for Optimal Zero Fluorescence

FIG. 7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530.8021
531.3026
931.5277
1053.5494
1055.5588
1056.5708
1058.5830
1059.5962
1060.6102
100
%
0
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
m/z

FIG. 8

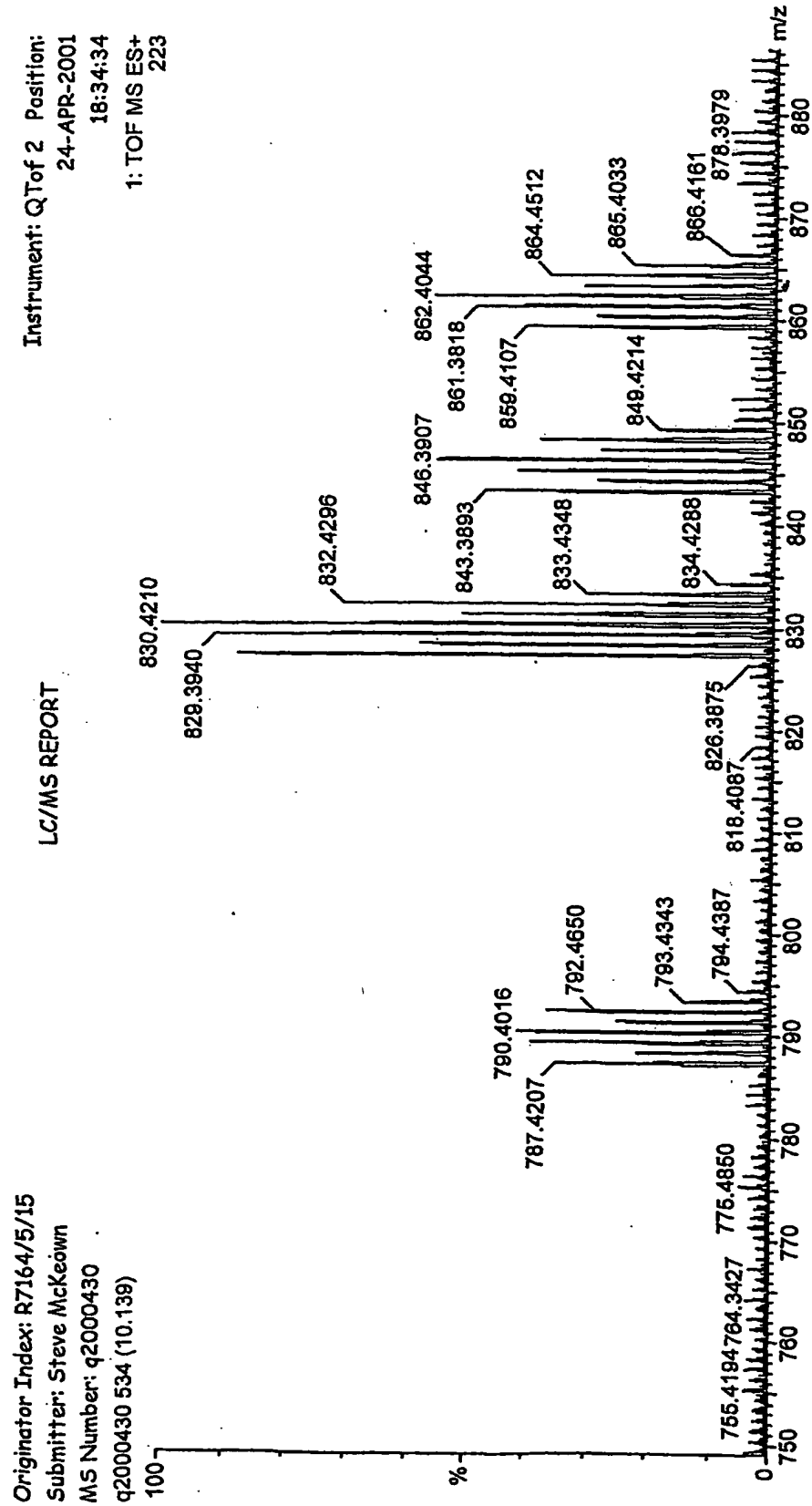

Originator Index: R7164/5/15
Submitter: Steve McKeown
MS Number: q2000430
q2000430 534 (10.139)
LC/MS REPORT
Instrument: QTof 2 Position:
24-APR-2001
18:34:34
1: TOF MS ES+
223
100
%
0
750
760
770
780
790
800
810
820
830
840
850
860
870
880
m/z
755.4194
764.3427
776.4850
787.4207
790.4016
792.4650
793.4343
794.4387
818.4087
826.3875
829.3940
830.4210
832.4296
843.3893
833.4348
834.4288
846.3907
861.3818
859.4107
849.4214
862.4044
864.4512
865.4033
866.4161
878.3979

FIG. 9

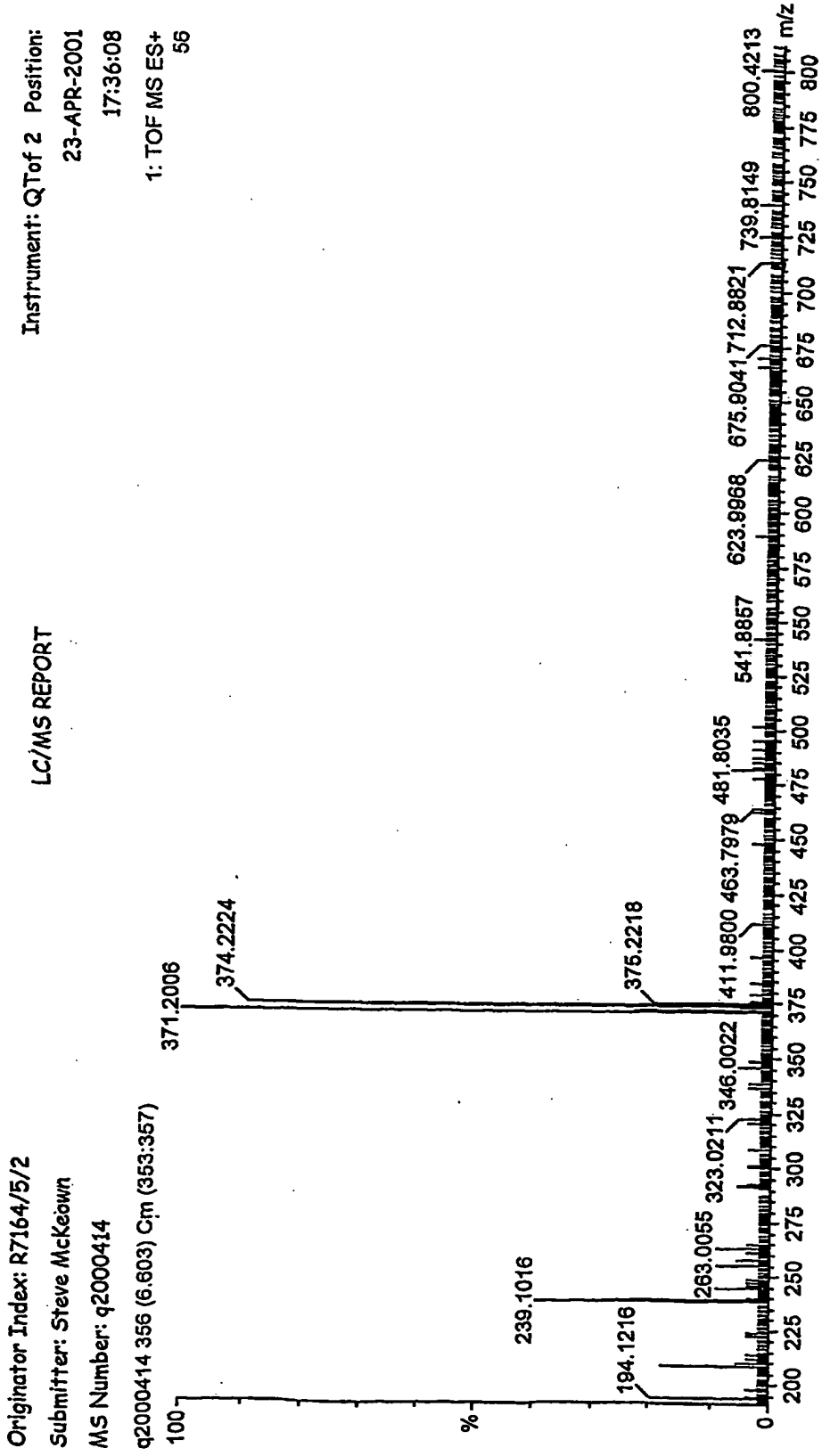

Originator Index: R7164/5/2
Submitter: Steve McKeown
MS Number: q2000414
q2000414 356 (6.603) Cm (353:357)
LC/MS REPORT
Instrument: QTof 2 Position:
23-APR-2001
17:36:08
1: TOF MS ES+
56
100
%
0
371.2006
374.2224
375.2218
411.9800
463.7979
481.8035
541.8857
623.9968
675.9041
712.8821
739.8149
800.4213
239.1016
194.1216
263.0055
323.0211
346.0022
200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 725 750 775 800
m/z

FIG. 10

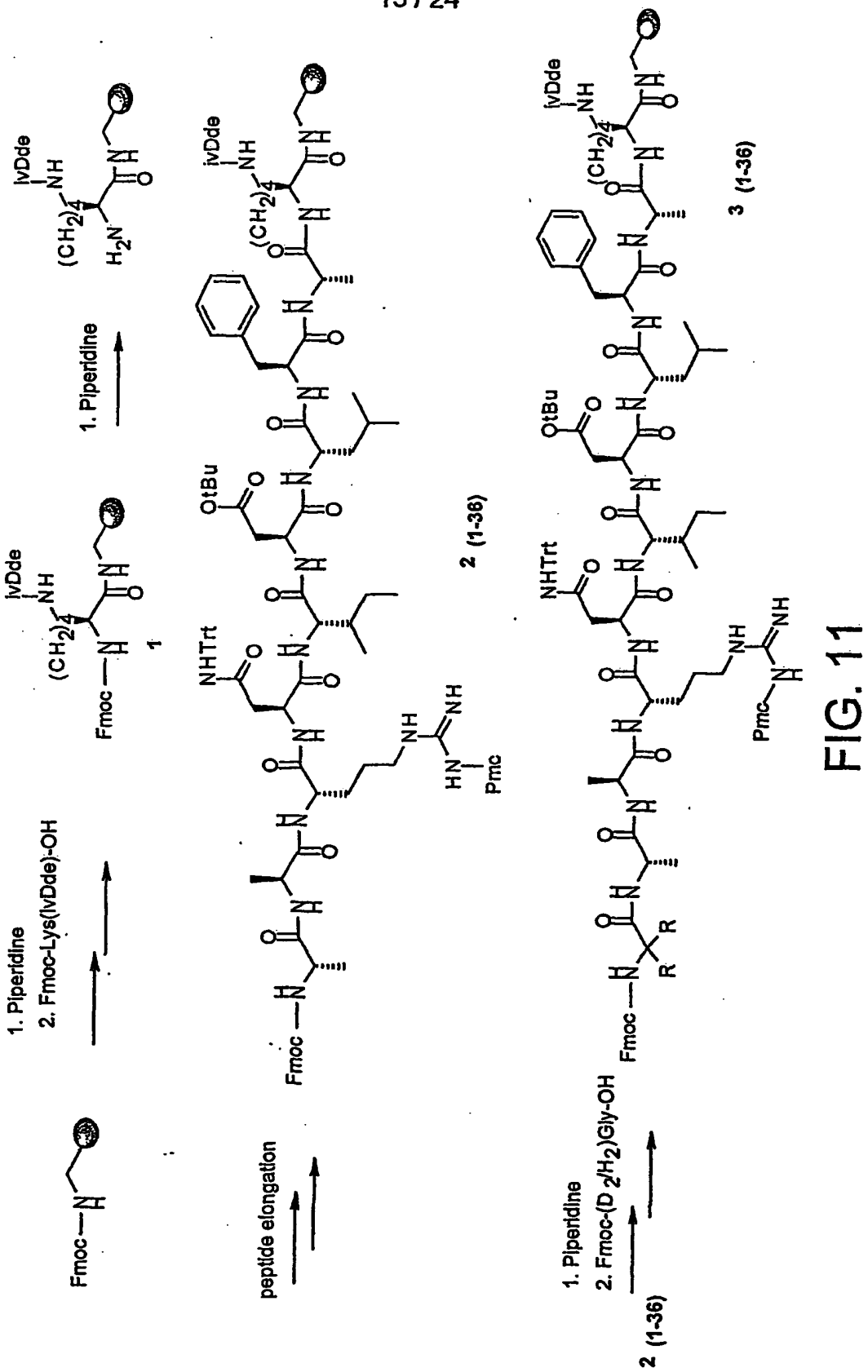

1. Piperidine
2. Fmoc-Lys(ivDde)-OH
peptide elongation
1. Piperidine
2. Fmoc-(D 2/H2)Gly-OH
2 (1-36)
1
2 (1-36)
3 (1-36)

FIG. 11

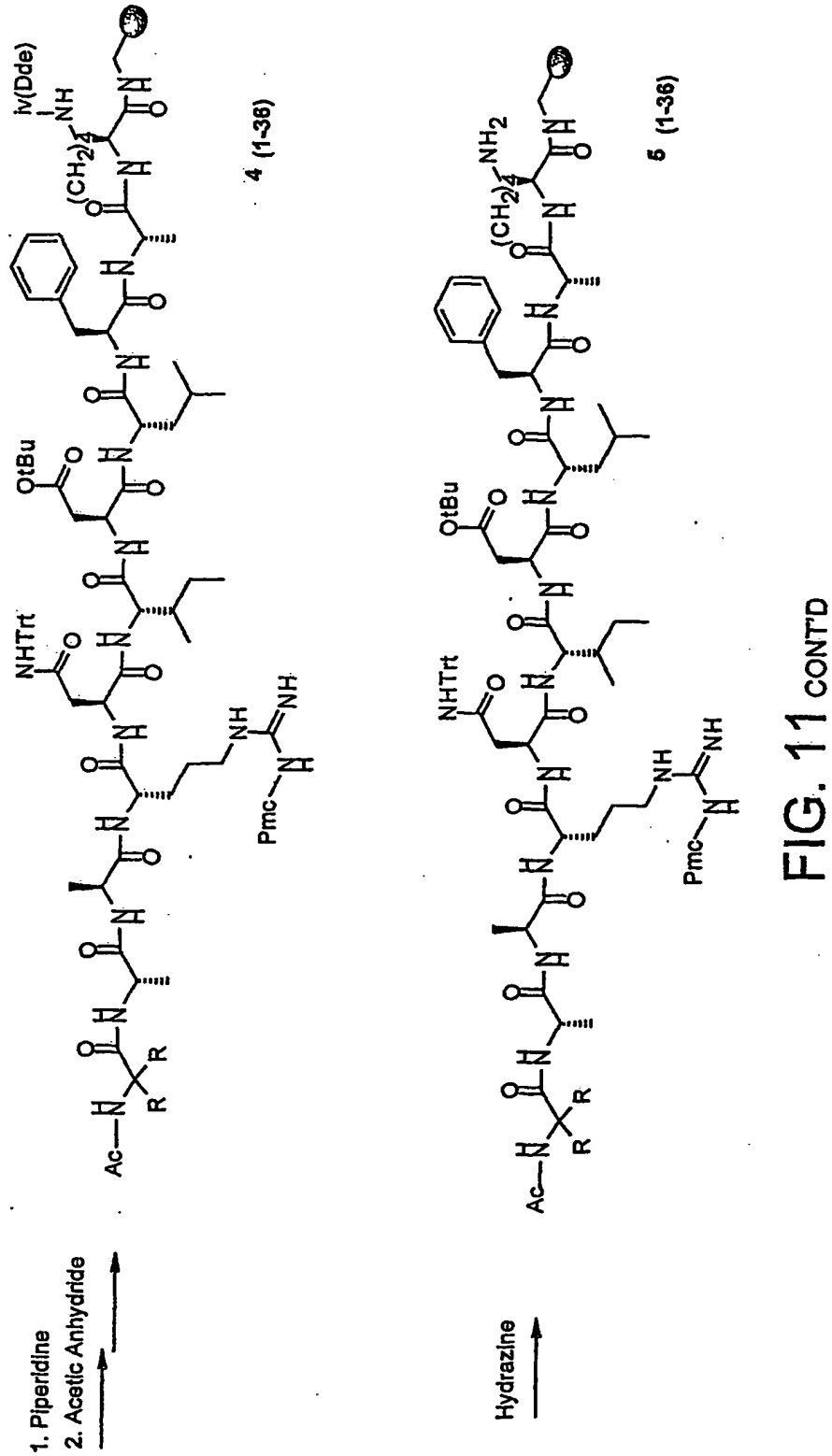

Iv(Dde)
4 (1-36)
OtBu
NHTrt
Pmc
Ac
1. Piperidine
2. Acetic Anhydride
5 (1-36)
Hydrazine

FIG. 11 CONT'D

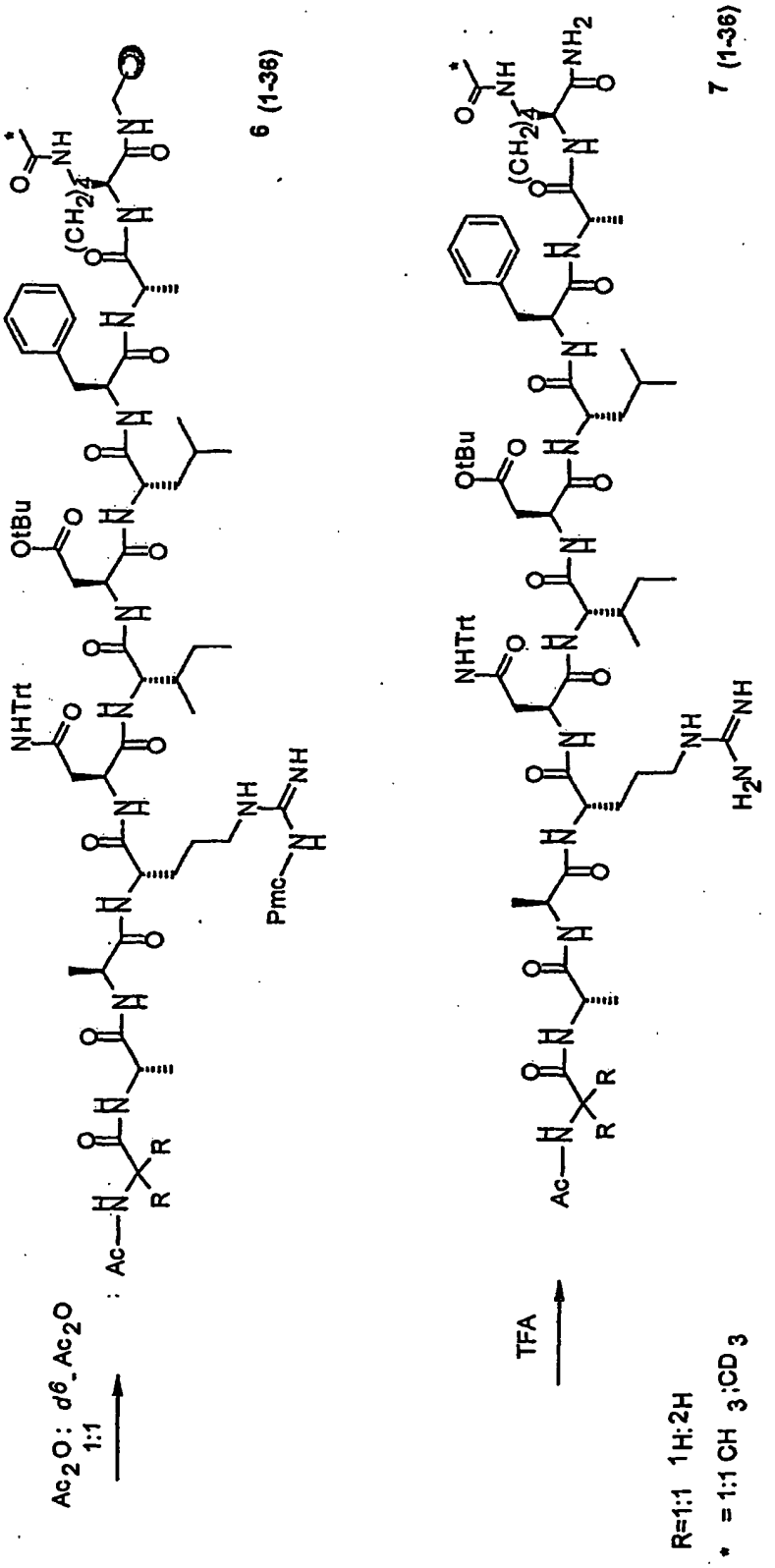


FIG. 11 CONT'D

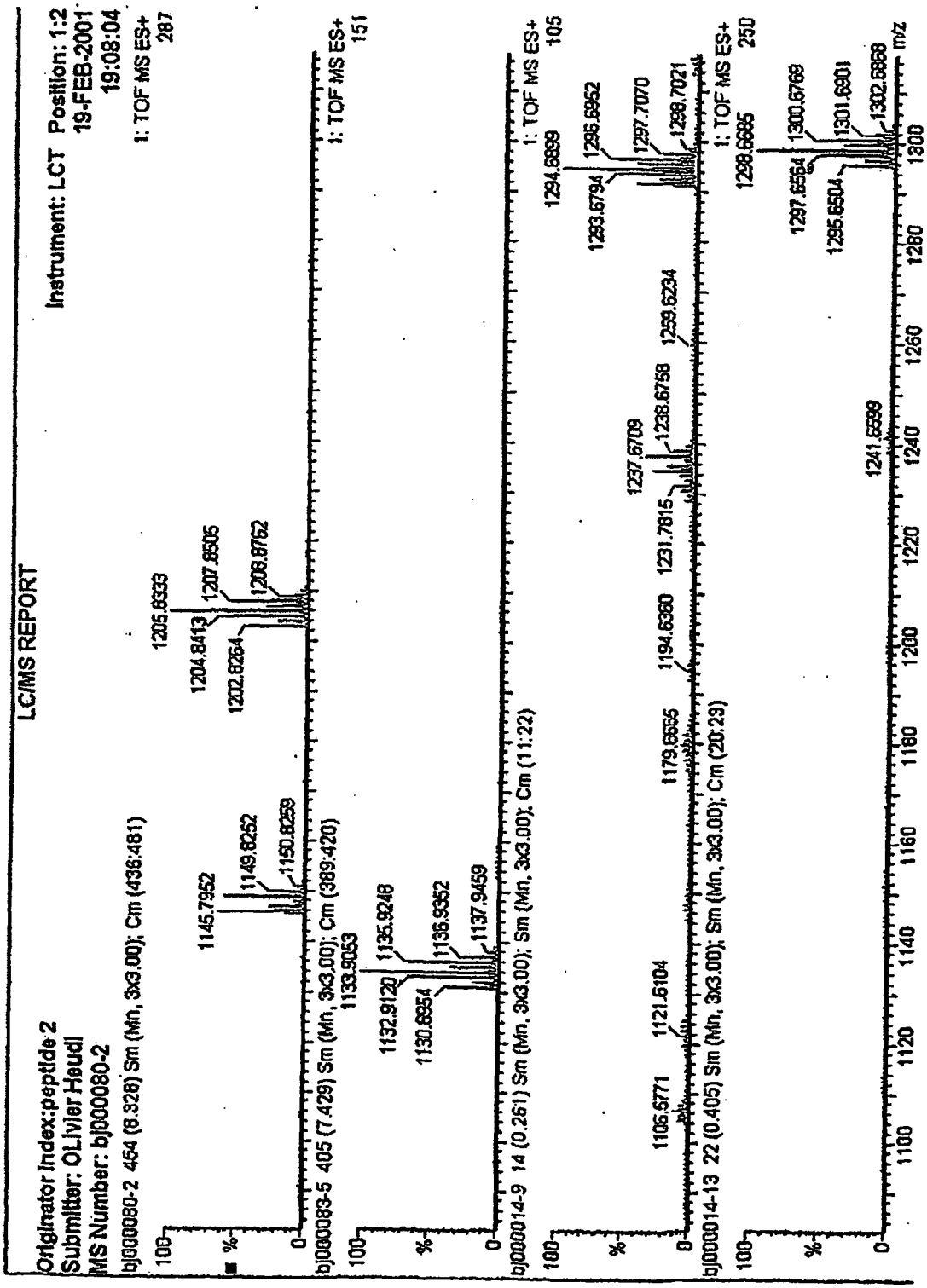

LC/MS REPORT
Originator Index:peptide 2
Submitter: OLivier Heudi
MS Number: bj000080-2
Instrument: LCT Position: 1:2
19-FEB-2001
19:08:04
bj000080-2 454 (8.328) Sm (Mn, 3x3.00); Cm (436:481)
1: TOF MS ES+ 287
1205.6333
1207.6505
1204.8413
1208.6762
1202.8264
1145.7952
1149.8252
1150.8259
bj000083-5 405 (7.429) Sm (Mn, 3x3.00); Cm (389:420)
1: TOF MS ES+ 151
1133.9053
1135.9248
1132.9120
1136.9352
1130.8854
1137.9459
bj000014-9 14 (0.261) Sm (Mn, 3x3.00); Cm (11:22)
1: TOF MS ES+ 105
1294.6899
1296.6952
1293.6794
1297.7070
1298.7021
1237.6709
1238.6758
1299.6234
1231.7815
1194.6360
1179.6665
1121.6104
1106.5771
bj000014-13 22 (0.405) Sm (Mn, 3x3.00); Cm (20:23)
1: TOF MS ES+ 250
1298.6685
1300.6769
1297.6554
1301.6901
1295.6504
1302.6868
1241.6899
1100
1120
1140
1160
1180
1200
1220
1240
1260
1280
1300
m/z

FIG. 12

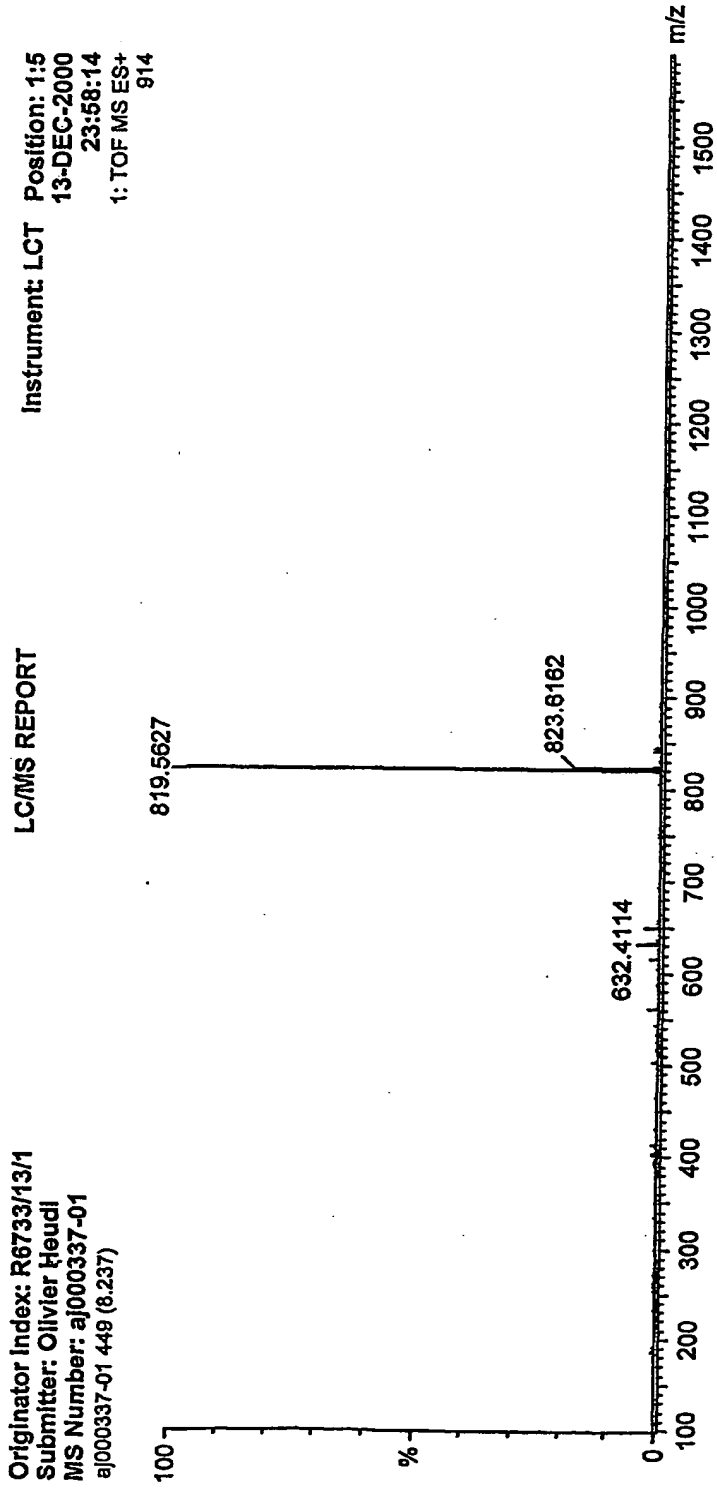

Originator Index: R6733/13/1
Submitter: Olivier Heudi
MS Number: aj000337-01
aj000337-01 449 (8.237)
LC/MS REPORT
Instrument: LCT Position: 1:5
13-DEC-2000
23:58:14
1: TOF MS ES+
914
819.5627
823.8162
632.4114
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

FIG. 13a

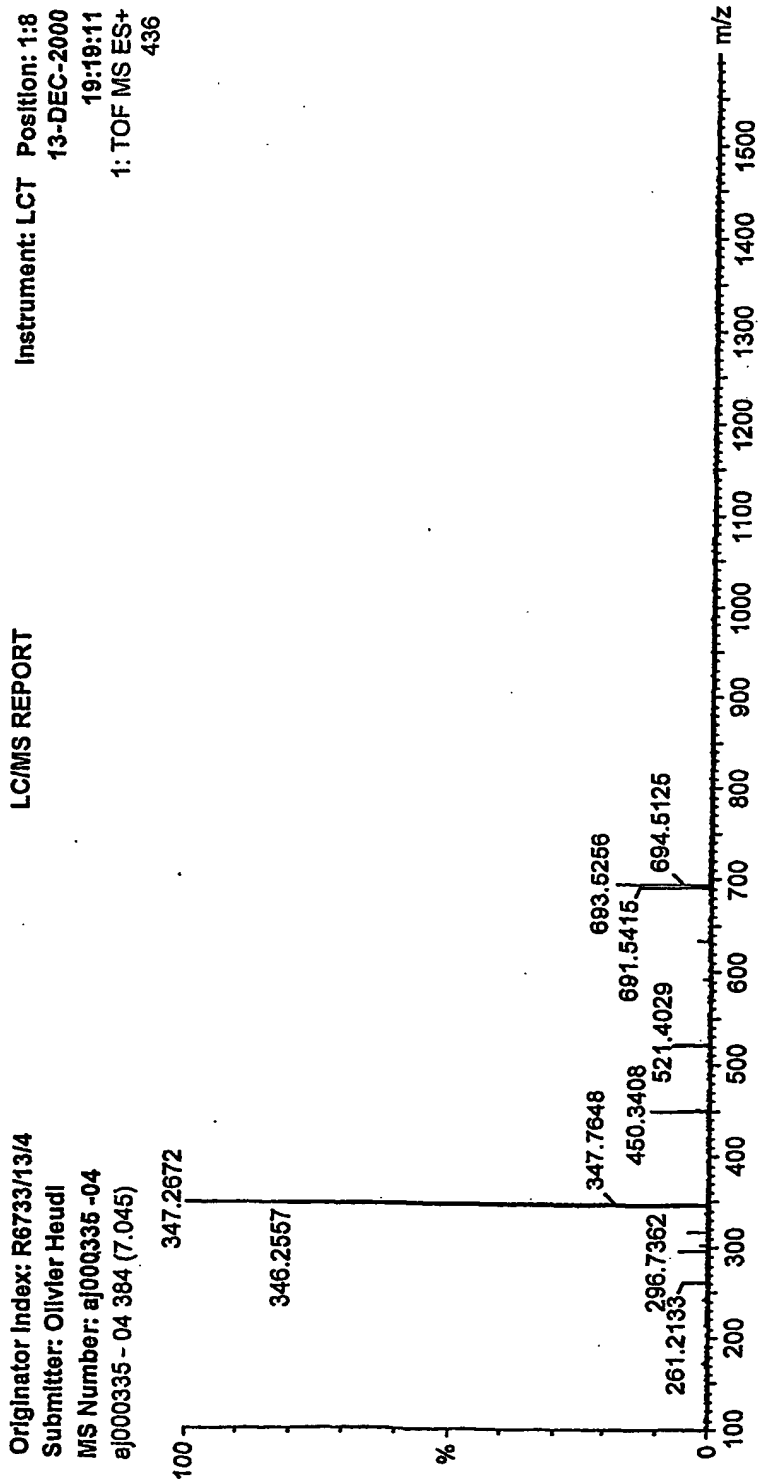

Originator Index: R6733/13/4
Submitter: Olivier Heudi
MS Number: aj000335 -04
aj000335 - 04 384 (7.045)
LC/MS REPORT
Instrument: LCT Position: 1:8
13-DEC-2000
19:19:11
1: TOF MS ES+
436
347.2672
346.2557
347.7648
450.3408
521.4029
691.5415
693.5256
694.5125
296.7362
261.2133
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

FIG. 13b

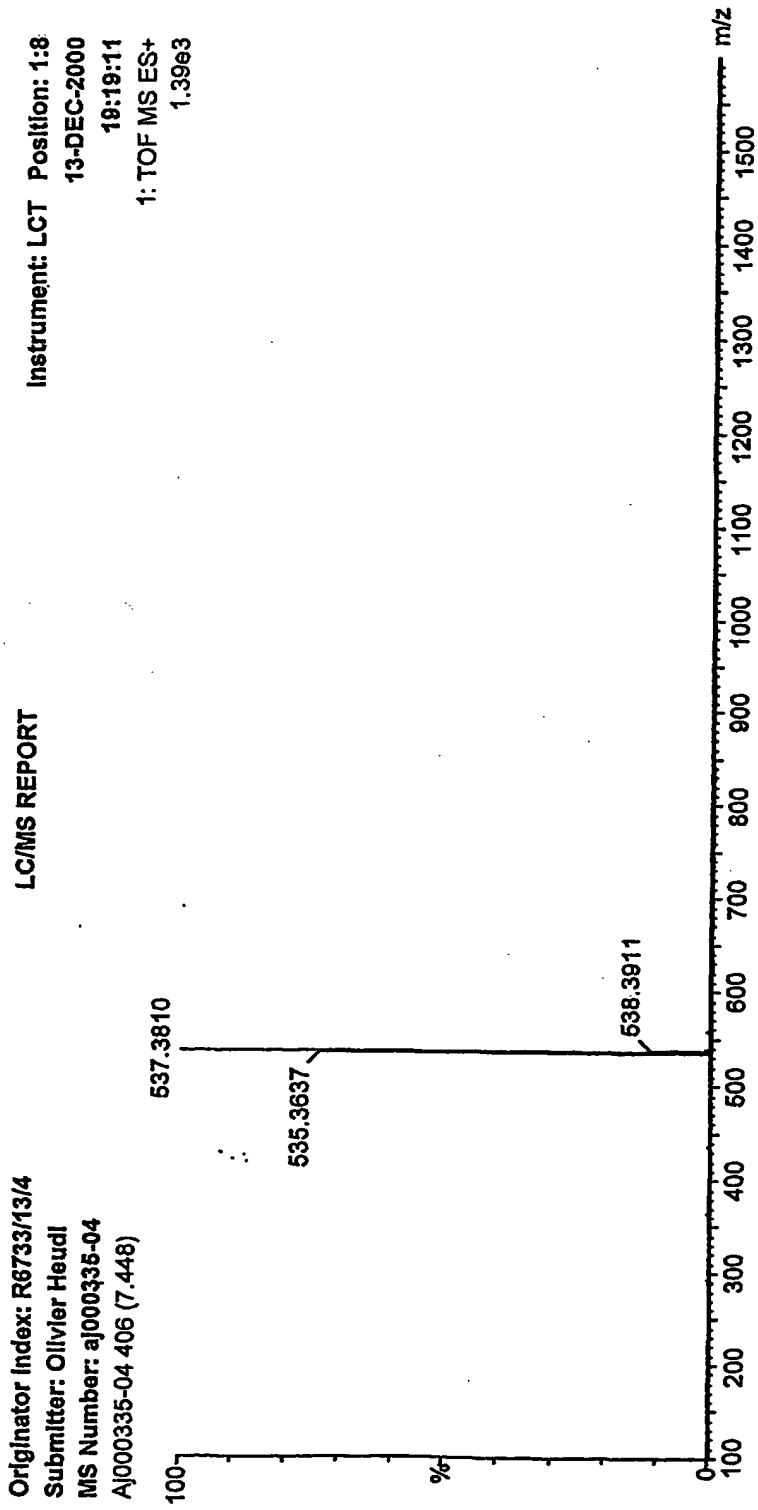

Originator Index: R6733/13/4
Submitter: Olivier Heudi
MS Number: aj000335-04
Aj00335-04 406 (7.448)
LC/MS REPORT
Instrument: LCT Position: 1:8
13-DEC-2000
19:19:11
1: TOF MS ES+
1.39e3
537.3810
535.3637
538.3911
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

FIG. 13c

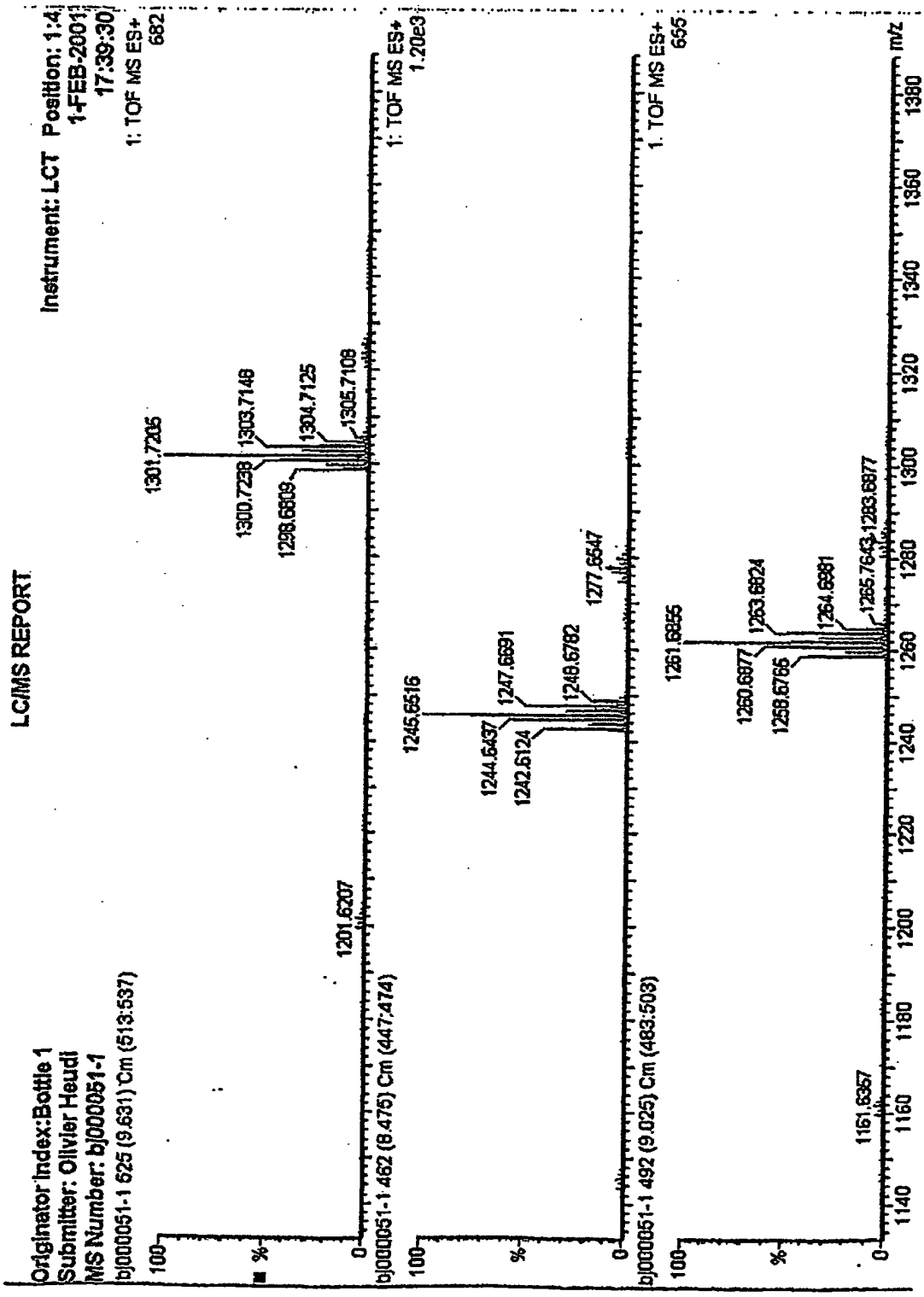

LC/MS REPORT
Originator Index:Bottle 1
Submitter: Olivier Heudi
MS Number: bj000051-1
Instrument: LCT Position: 1:4
1-FEB-2001
17:39:30
bj000051-1 625 (9.631) Cm (513:537)
1: TOF MS ES+
682
1301.7205
1303.7148
1304.7125
1305.7108
1300.7238
1298.6809
1201.6207
bj000051-1 462 (8.475) Cm (447:474)
1: TOF MS ES+
1.20e3
1245.6516
1247.6691
1244.6437
1248.6782
1242.6124
1277.6547
bj000051-1 492 (9.025) Cm (483:503)
1: TOF MS ES+
655
1261.6855
1263.6924
1260.6877
1264.6981
1258.6765
1265.7643
1283.6877
1161.6357
m/z
%
100
0
1140
1160
1180
1200
1220
1240
1260
1280
1300
1320
1340
1360
1380

FIG. 14

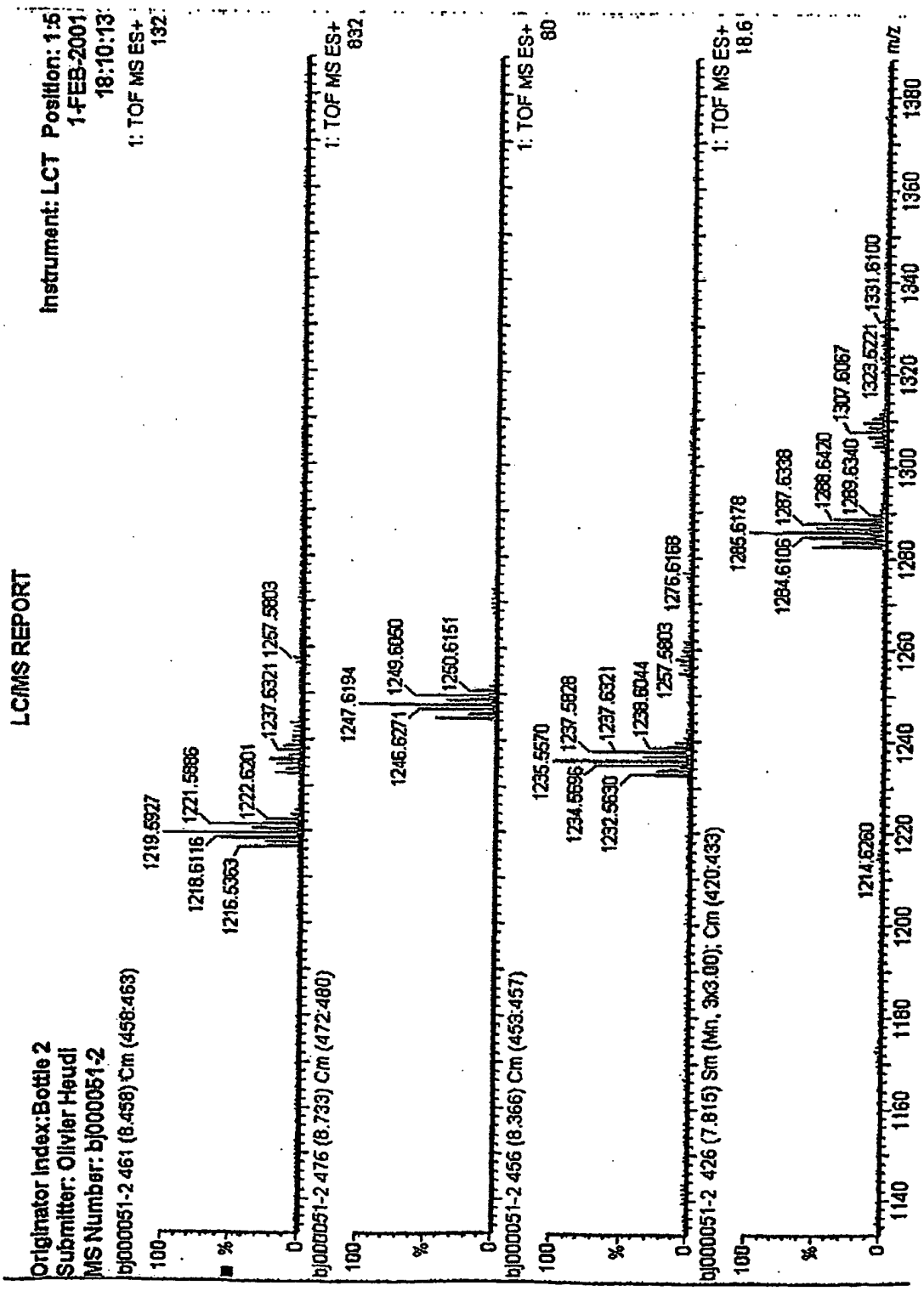

LCMS REPORT
Originator Index:Bottle 2
Submitter: Olivier Heudi
MS Number: bj000051-2
Instrument: LCT Position: 1:5
1-FEB-2001
18:10:13
bj000051-2 461 (8.458) Cm (458:463)
1: TOF MS ES+
132
1219.5927
1221.5986
1218.6116
1222.6201
1216.5363
1237.6321
1257.5803
bj000051-2 476 (8.733) Cm (472:480)
1: TOF MS ES+
832
1247.6194
1249.6050
1246.6271
1250.6151
bj000051-2 456 (8.366) Cm (453:457)
1: TOF MS ES+
80
1235.5570
1237.5828
1234.5596
1237.6321
1232.5630
1238.6044
1257.5803
1276.6168
bj000051-2 426 (7.815) Sm (Mn, 3x3.00); Cm (420:433)
1: TOF MS ES+
18.6
1285.6178
1287.6338
1284.6105
1288.6420
1289.6340
1307.6067
1323.6221
1331.6100
1214.6260
%
100
0
1140
1160
1180
1200
1220
1240
1260
1280
1300
1320
1340
1360
1380
m/z

FIG. 15

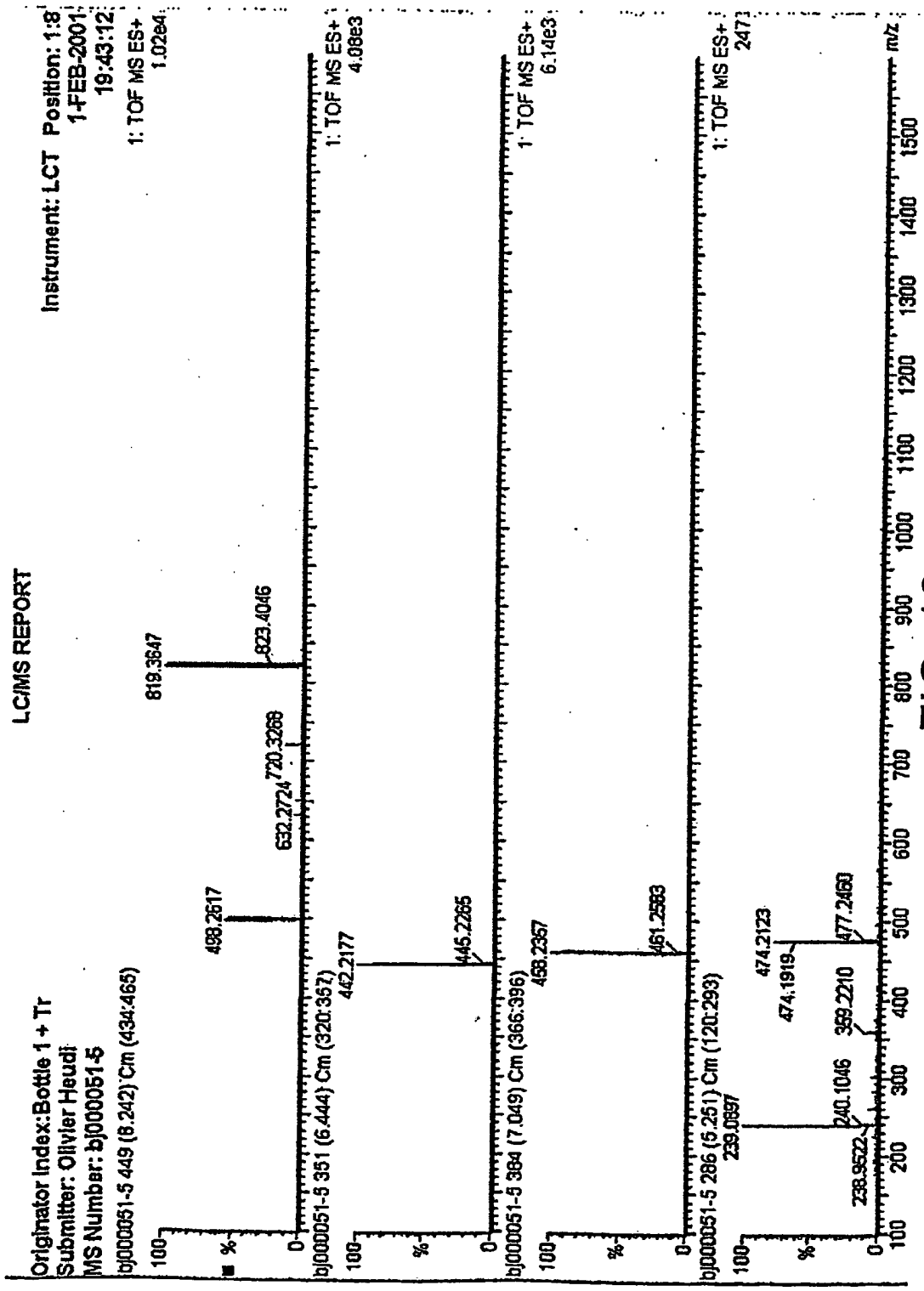


FIG. 16

BEST AVAILABLE COPY

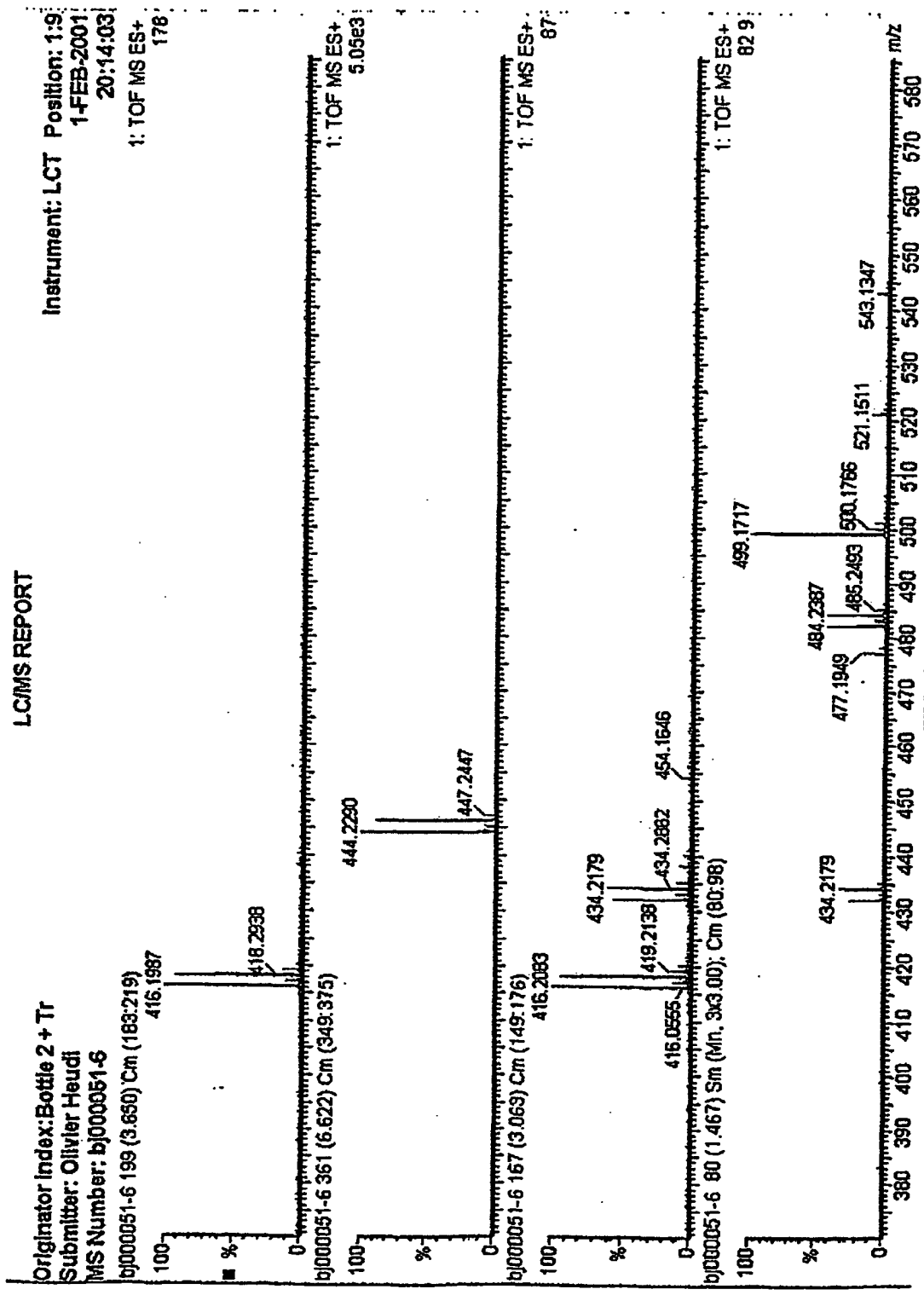
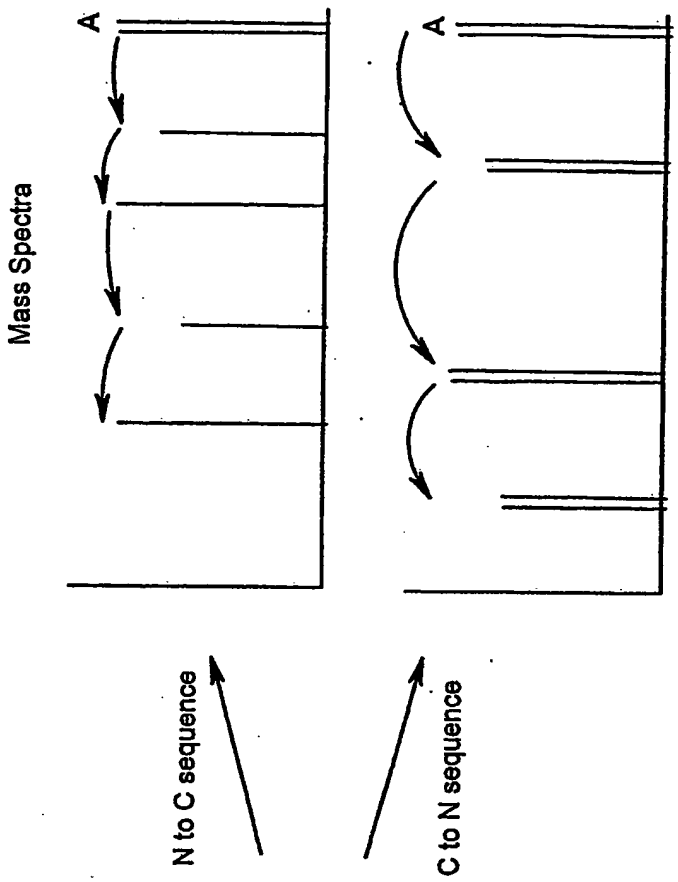


FIG. 17

BEST AVAILABLE COPY


A
A
Mass Spectra
N to C sequence
C to N sequence

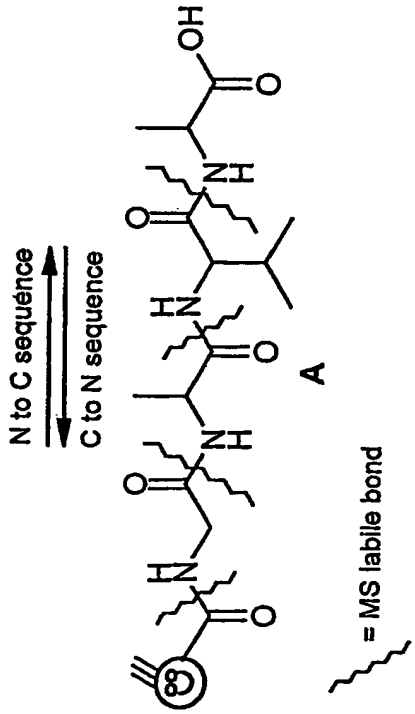

OH
O
NH
O
NH
O
A
NH
O
NH
O
N to C sequence
C to N sequence
= MS labile bond

FIG. 18

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/GB 02/02921

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/532

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEN X ET AL: "Site-specific mass tagging with stable isotopes in proteins for accurate and efficient protein identification" ANALYTICAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. COLUMBUS, US, vol. 72, no. 6, 15 March 2000 (2000-03-15), pages 1134-1143, XP002207456 ISSN: 0003-2700 abstract ---	1-30
A	WO 99 60007 A (ISIS INNOVATION LTD (GB)) 25 November 1999 (1999-11-25) abstract --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2002	21.10.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moa Emling Grönkvist

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int nal Application No
PCT/GB 02/02921

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 338 686 A (HELLERSTEIN MARC K) 16 August 1994 (1994-08-16) the whole document -----	1-30

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inta tal Application No
PCT/GB 02/02921

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9960007 A	25-11-1999	AU 3943799 A	06-12-1999
		EP 1068216 A2	17-01-2001
		WO 9960007 A2	25-11-1999
		JP 2002515588 T	28-05-2002
US 5338686 A	16-08-1994	NONE	